Genes Controlling Early Seed Development in *Arabidopsis*

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Statement of Originality

This thesis contains no material which has been accepted for the award of any other degree in any university or other tertiary institution and, to the best of my knowledge and brief, contains no material previously published by another person, except where due reference has been made in the text.

Ming Luo

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ABSTRACT

FIS class genes, *MEA/FIS1*, *FIS2*, and *FIE/FIS3* have specific functions in *Arabidopsis thaliana* seed development. The *FIS* class genes prevent the central cell nucleus from developing into endosperm before fertilisation and regulate endosperm proliferation after fertilisation. The loss of function of any of the *FIS* genes causes the failure of repression of division of the central cell nucleus and leads to diploid autonomous endosperm growth in unfertilised ovules and endosperm over-proliferation and embryo arrest in fertilised ovules.

The predicted protein sequences encoded by these three genes show that MEA/FIS1, FIE/FIS3 and FIS2 are related to polycomb group proteins, Enhancer of zeste, Extra sex combs and Suppresser of zeste 12 previously described in Drosophila. In this study, the expression pattern of each of the *FIS* class genes was investigated by using a promoter::reporter gene (*GUS*) system. *FIS1/MEA::GUS*, *FIS2::GUS* and *FIE/FIS3::GUS* expression was detected in the unfertilised central cell nucleus and in the dividing endosperm nuclei in the endosperm. *FIS* gene expression in the unfertilised central cell nucleus is consistent with its role in repressing endosperm development. The expression in dividing endosperm also suggests a role in endosperm development because the endosperm is over-proliferated in all three mutants. *FIE/FIS3* activity can be detected in other sporophytic tissues, suggesting that *FIE/FIS3* may have functions other than in endosperm. *MEA/FIS1, FIS2* and *FIE* were shown to be imprinted; during early seed development only the maternal allele was expressed.

The over proliferation of the *mea/fis1*, *fis2* and *fie/fis3* endosperm is reduced by fertilising the *fis* ovules with hypomethylated pollen without activating the paternal *FIS* copies of these three genes. Three paternal modifiers for *fis2* and two paternal modifiers for *mea/fis1* regulated by DNA methylation were identified.

In another part of this study, two mutants (*min2* and *min3*) with reduced seed size were characterised cytologically. Precocious endosperm cellularisation and reduction of endosperm content were the primary causes of seed size reduction in the mutants. *MIN3* encodes *WRKY10*, a *WRKY* class transcription factor (Eulgem et al., 2000). *MIN3* promoter::*GUS* fusions show expression of the gene is restricted to pollen, and the developing endosperm from the two nucleate stage at ~12 hour post fertilization to endosperm cellularisation at ~96 hours. The early endosperm expression of *MIN3* is independent of parent of origin showing that the gene is not imprinted. There is no expression in an unpollinated female gametophyte. *MIN3* is also expressed in the globular embryo but has no embryo expression at the late heart stage of development. The *MIN3* promoter has three W-boxes, the binding motifs for the WRKY protein, implying auto-regulation. The *MIN3*::*GUS* transgene has a higher level of expression in *min3/min3* plants than in *MIN3/MIN3* plants suggesting that *MIN3* is a negative regulator of its own expression.

CHAPTER 1

General introduction

The reproductive process in plants is an important area in biology, which still requires substantial genetic and molecular investigation to elucidate the complex process. Most food is in the form of seeds, which are the products of fertilization. Angiosperms, or higher plants, have a unique seed forming process, double fertilization: the egg cell is fused with a sperm nucleus and the central cell fuses to another sperm nucleus both coming from the same pollen grain. This leads to the formation of the embryo and endosperm (reviewed by Chaudhury et al., 2000; Drews et al., 1998; Leonore et al., 1993). Double fertilization occurs in the female gametophyte, also referred as the embryo sac or megagametophyte, which normally is a seven-celled structure derived from the megaspore mother cell via meiosis, spore selection and mitosis. The embryo sac is embedded within the sexual organs of the flower (Fig. 1.1). After double fertilization, the fertilized egg or zygote develops into an embryo with genetically controlled pattern formation (Jurgens et al., 1998); the fertilized central cell which develops into the endosperm also displays developmental programming.

In the developing embryo the fate of each cell is determined in a position-dependent manner to form a new functional plant (reviewed by Jurgens et al., 1998). *Arabidopsis* embryo development follows a series of stages identified as zygote, globular, heart, torpedo and walking stick. The basic body plan is completely established when the embryo reaches heart stage (Fig.1. 2). Mutations affecting the body plan or interrupting pattern formation have been described in *Arabidopsis*.

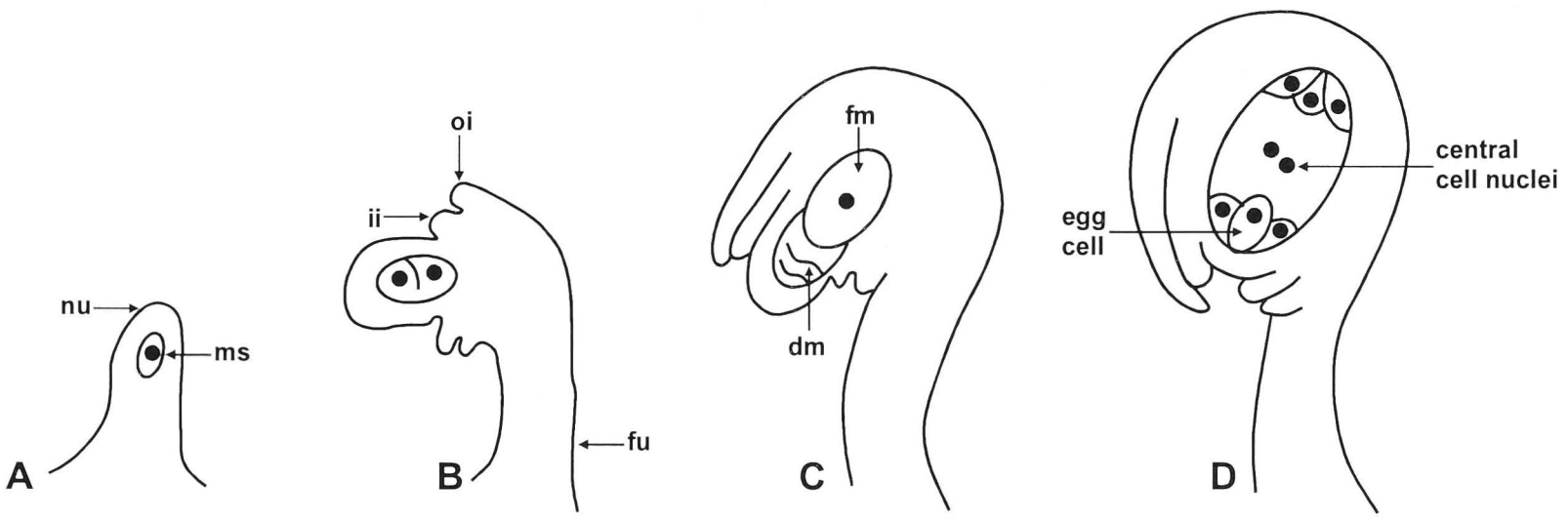


Fig. 1.1 Ovule development. (A) Ovule showing a single megasporocyte (ms) and nucellus (nu). (B) The megasporocyte has undergone the first meiosis. The inner integument (ii) and outer integument (oi) have initiated. (C) After meiosis one functional megaspore (fm) at the chalazal end expanded, other three become degenerate. Dm, degenerated megaspores. (D) Ovule after megagametogenesis. The mature embryo sac contains seven cells and eight nuclei.

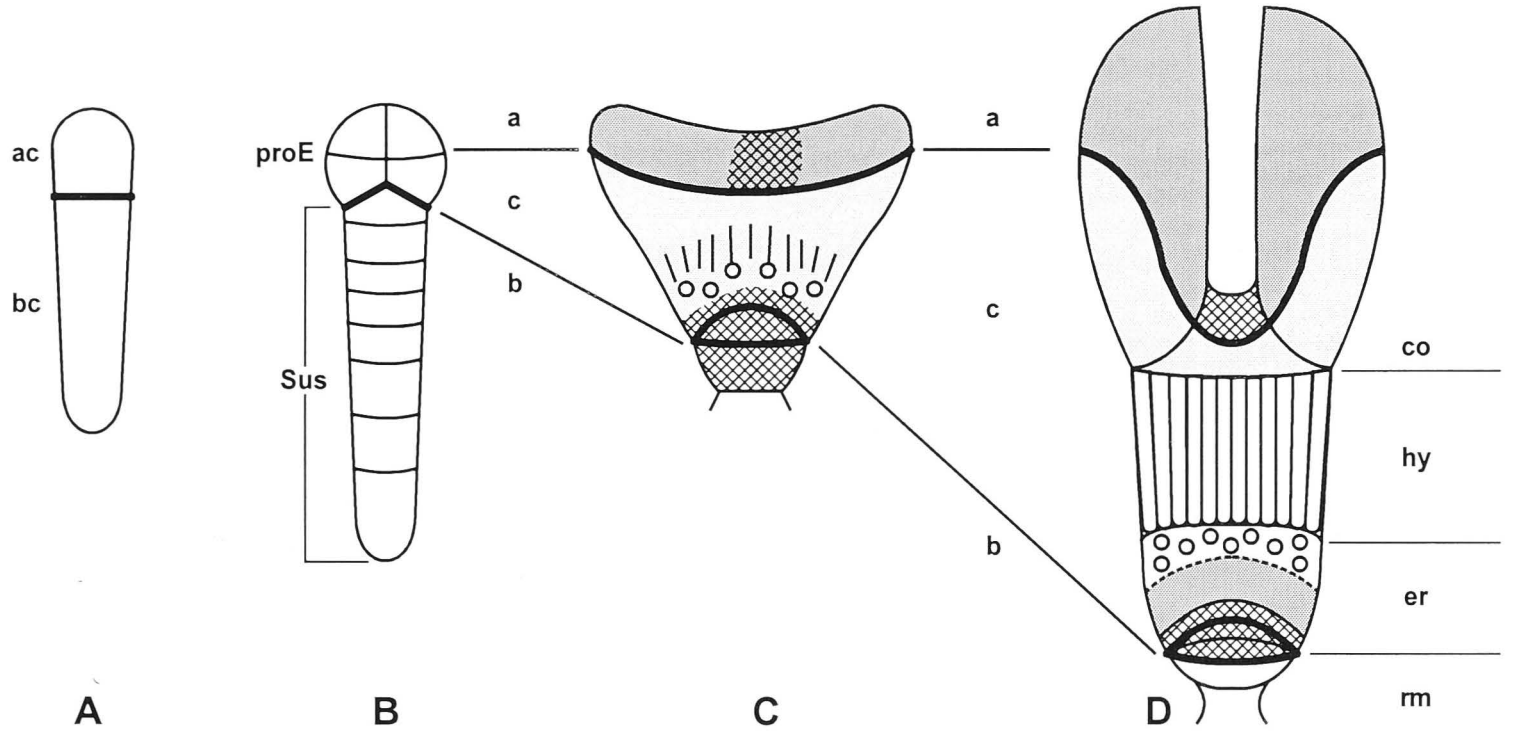


Fig. 1.2 Development of the *Arabidopsis* embryo. (A) Asymmetric division of the zygote. ac, apical cell; bc, basal cell. (B) Eight-cell stage. The proembryo (proE) consists of two tiers of each four cells (a, c). sus, suspensor; a, apical; c, central; b, basal region. (C) Heart stage embryo. (D) Torpedo stage embryo. Primordia of seedling structures: co, cotyledons; hy, hypocotyl; er, embryonic root; RM, root meristem; SM, shoot meristem.

The endosperm provides nutrition to the growing embryo. The endosperm is not a uniform structure. The early syncytial endosperm in *Arabidopsis* is divided into three mitotic domains possibly serving different functions (Boisnard-Lorig et al., 2001).

Contributions to early development of the endosperm are different between the maternal genome and paternal genome (Lin et al., 1984; Scott et al., 1998; Kinoshita et al., 1999). It has been speculated from work in *Arabidodpsis* that the paternal genome is not important for the early embryo and endosperm development, because a broad range of paternally derived genes with distinct functions are silenced in the early seed, indicating that it is mainly under maternal control (Vielle-Calzada et al., 2000). The identification and isolation of *FIS* genes and mutants in *Arabidopsis* has shown that the central cell is repressed by the FIS protein complex, homologs of Polycomb group proteins initially identified as chromatin modifiers repressing homeotic genes in *Drosophila* (Grossniklaus et al., 1999; Luo et al., 1999; Ohad et al., 2000). By removing the FIS function, the central cell develops autonomously into endosperm.

In some plant species, meiosis during megegametogenesis and fertilization are by-passed, leading to the formation of an unreduced embryo sac and autonomous seed setting, a process called apomixis (Grimanelli et al., 2001). In sexual plants, the egg cell and central cell will not develop into embryo and endosperm until fertilization brings the paternal and maternal genome with their differentially expressed genes together, indicating that genomic imprinting is a possible barrier that prevents the egg and central cell from developing into the embryo and endosperm. An apomictic species must overcome the parental imprinting

barrier, leading to embryo and endosperm formation without involving the paternal genome. By identifying the important imprinted genes involved in embryo and endosperm development, the mechanism controlling apomixis may be elucidated.

Understanding seed development is also important for agricultural productivity. Seed represents an important source of food, feed, and industrial raw material for mankind. Seed size is one of the significant factors affecting crop production. Endosperm accounts for most of the bulk of cereal seed and determines the seed size. Even in *Arabidopsis*, a eudicot, where the endosperm is a transient tissue, seed size is affected by the number of endosperm nuclei (Scott et al., 1998). Understanding the early events related to endosperm development remains a challenge.

1.1 Female gametophyte formation and double fertilization

Over 15 different types of female gametophyte formation have been described. In general, female gametophyte development follows two phases: megasporogenesis and megagametogenesis. During megasporogenesis, a diploid megaspore mother cell in the somatic nucellus undergoes meiotic divisions and four megaspore nuclei are produced. Subsequent mitotic divisions, nuclear migration, and cytokinesis during megagametogenesis produce the mature embryo sac. Different patterns of embryo sac formation are observed among plant species. Figure 1.3 shows some of the modes in embryo sac formation.

Among them, the most common pattern displayed in plant species, including *Arabidopsis* and rice is the so-called Polygonum type illustrated in Fig. 1.3. During megasporogenesis, a diploid megaspore mother cell in

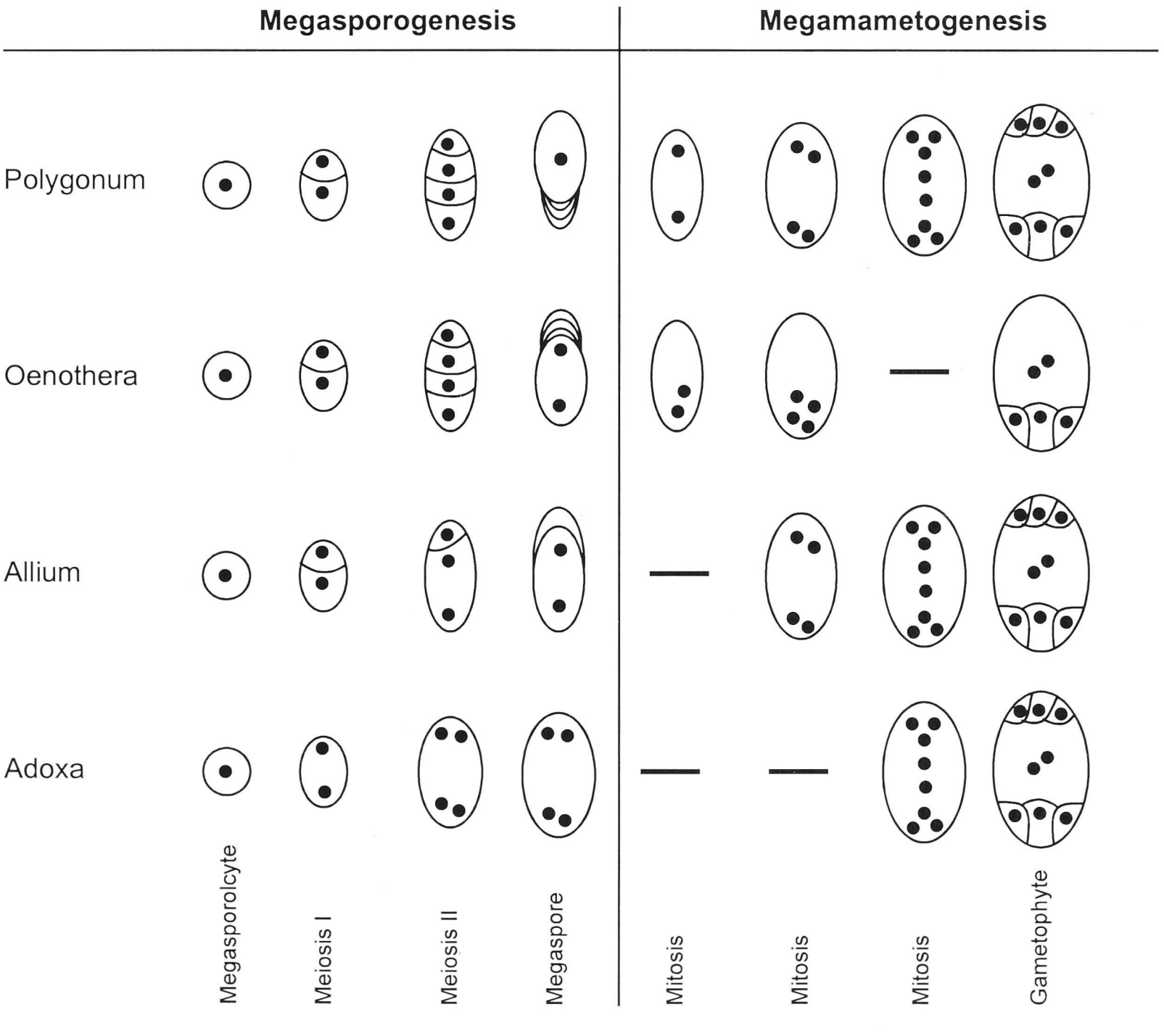


Fig 1.3 Patterns of embryo sac development are illustrated schematically.

the somatic nucellus undergoes meiotic divisions to form four haploid megaspores. Three of these close to the micropylar end degenerate and the chalazal-most one survives. After megasporogenesis, the surviving megaspore undergoes three rounds of mitosis to produce an eight-nucleate cell. Two nuclei, one from each pole, migrate to the central region of the cell to become the central cell nucleus. In *Arabidopsis*, the two central cell nuclei fuse to form the one diploid nucleus before fertilization. In some species such as maize this fusion happens after fertilization. During polar nuclei migration, the embryo sac cellularises to form a seven-celled structure. The three cells in the micropylar region become the egg apparatus including the egg cell and two synergids; the three cells at the chalazal end develop into antipodals which degenerate in *Arabidopsis* (Schneitz et al., 1995), or proliferate in species such as maize (Vollbrecht and Hake., 1995). The central region forms a central cell with the fused diploid nucleus or two haploid nuclei. Each cell type displays a different form of structural specialization. In *Arabidopsis*, the egg apparatus cells are highly polarized; the egg has a large micropylar vacuole and chalazally sited nucleus. The central cell is long and vacuolated with a large diploid nucleus. Vacuoles are absent in synergids (Mansfield et al., 1991). Upon reaching this stage the embryo sac is ready for fertilization.

Double fertilization is not restricted to angiosperms. In a relative of the angiosperms, the *Gnetales*, a non-flowering seed plant, there is a regular process of double fertilization that yields two diploid embryos (Friedman, 1998). However, the double fertilization in angiosperms results in the formation of the embryo and endosperm. It has been postulated that in angiosperms the endosperm is derived from a twin embryo as seen in the *Gnetales*.

At maturity, pollen grains house two sperm cells and the vegetative cell. Once the pollen tube reaches one of the synergids in the embryo sac, the male gametes are released from the cytoplasm of the tube into the embryo sac. In cotton the synergid that receives the pollen tube displays special structures; at one end of the cell, the cell wall is elaborated in a filiform apparatus, while the wall at the opposite end disappears (reviewed by Jensen, 1998). The chalazal wall of the highly polarized egg cell also disappears. These structures obviously facilitate the penetrence of the pollen tube. Upon fertilization, the pollen tube grows through the filiform apparatus into the degenerating cytoplasm and discharges the gametes. The breakdown of the plasma membrane of the synergid helps the sperm plasma membrane come into direct contact with the egg and central cell plasma membrane. Then the sperm nuclei are released into the egg cell and central cell to fuse with their nuclei. Whether particular male gametes are programmed to fertilize either the egg or the central cell is a subject of debate (Jensen, 1998).

1.2 Embryo and endosperm development

1.2.1 Embryogenesis

Embryogenesis describes the subsequent period of development, during which the zygote undergoes a complex series of morphological and cellular changes resulting in the formation of the mature embryo. The embryo is comprised of three domains: the apical domain including cotyledons and the shoot apex (meristem), the central domain consisting of the bulk of the axis (hypocotyl), and the basal domain including the root apex (meristem) (Fig. 1.2).

The embryo sac, egg cell and polar central cell appear polarized in many higher plant species (reviewed by Laux et al., 1997). The apical and basal axes are aligned to the chalaza-micropyle axis, indicating an orienting influence of the surrounding maternal tissue. Homozygous maternal effect mutations in *sin-1* and *sin-2* cause alterations in the embryo pattern, resulting in a symmetrical deletion of the embryonic axis and decreased embryo viability (Ray et al., 1996). The *SIN1* gene encodes a multi-domain protein and has sequence similarity to the Drosophila melanogaster gene Dicer, which encodes a multidomain ribonuclease specific for double-stranded RNA, first identified by its role in RNA silencing. The Dicer protein is essential for temporal control of development in animals, through the processing of small RNA hairpins that in turn inhibit the translation of target mRNAs (Golden et al., 2002). The SIN1 product might be involved in the processing of critical RNA involved in embryonic pattern formation.

The first phase of embryo development is the establishment of the body plan during the first one third of embryogenesis. Subsequent events include further growth of the embryo, further cell differentiation, and preparation for dormancy. In *Arabidopsis*, the first division of the zygote is asymmetric by which a smaller apical cell and a larger basal cell are formed. At the octant stage, the apical cell has given rise to four upper-tier and four lower-tier cells, and the basal cell has generated the hypophysis and the suspensor. At the heart stage, the apical domain that derived from the upper-tier cells, has been partitioned into cotyledon and shoot meristem primordia. The central domain, which derived from the lower-tier cells, has been subdivided into the upper-lower and the lower-lower tiers. At this stage the basic body plan of *Arabidopsis* embryo has been established. The lower-lower tier cells give rise to the hypocotyls,

root and initials of the root meristem. The basal domain derived from the hypophysis has formed the quiescent center of the root meristem and the initials of the central root cap.

Of the *Arabidopsis* embryonic pattern mutants analyzed, only mutations in the *GNOM* gene affect the apical-basal polarity of the embryo. The *gnom* mutant zygote does not elongate to the same extent as wild type and the first division appears symmetrical. Other phenotypes include lack of root and reduced apical structure. *GNOM* encodes a guanine nucleotide exchange factor that acts on an ADP ribosylation factor ARF-type G protein and is required for coordination of cell polarity along the apical-basal axis (Busch et al., 1996). Mutations in the *GURKE* gene specifically affect the apical domain (Torres-Ruiz et al., 1996.). Strong *gk* alleles abolish apical structures and lead to the formation of a disorganized green mass of cells at the apical end of *gk* seedlings. The central domain is also affected by *gk* mutation that result in shorter hypocotyls suggesting that the proper development of the apical domain requires *GK* activity.

Other genes controlling *Arabidopsis* embryonic patterning include *CUP1* (*CUP-SHAPED COTYLEDON1*), *CUP2*, *PIN-FORMED1* and *MONOPTEROS*. The *CUP1* and *2* genes are functionally redundant and required for both shoot apical meristem initiation and suppression of growth at the cotyledon boundaries (Takada et al., 2001). When both of these genes are disrupted, ectopic growth occurs at the boundary, resulting in almost completely fused cotyledons surrounding the apex, suggesting a role of these genes in promoting organ separation at the boundaries. *CUC*1 gene encodes a NAC-domain protein highly homologous to *CUC*2. *CUC*1 mRNA was detected in the presumptive

shoot apical meristem during embryogenesis, and at the boundaries between floral organ primordia. Overexpression of *CUC*1 was sufficient to induce adventitious shoots on the adaxial surface of cotyledons. *PIN-FORMED1* (*PIN1*) encodes a putative auxin efflux carrier and *MONOPTEROS* (*MP*) encodes an Auxin Response factor. They were thought to mediate auxin signaling respectively (Aida et al., 2002). The corresponding mutants show similar defects in apical patterning, including cotyledon fusion and dissymmetric organ positioning. Both mutations perturb the spatial expression patterns of *CUC*1 and *CUC*2. Genetic analysis indicates that *PIN1* and *MP* are required for the activity of *CUC*2 while *CUC*1 activity is only slightly affected by both mutations. These results suggest a differential regulation of the *CUC* genes by *PIN1* and *MP*, indicating that *PIN1* and *MP* regulate apical patterning partially through the control of *CUC* gene expression.

Another class of embryo mutants alters the fate of embryonic cells. The *Arabidopsis LEAFY COTYLEDON* genes, *LEC1*, *LEC2* and *FUSCA3*, play key roles in controlling embryo development (Lotan et al., 1998; Stone et al., 2001). Unlike most other embryonic regulators that function during specific stages of embryogenesis, *LEC* genes are unique in that they are required to specify suspensor cell fate and cotyledon identity. *LEC* genes are also needed during the maturation phase for acquisition of desiccation tolerance and suppression of germination. The *LEC1* gene encodes a HAP3 subunit of the CCAAT box-binding transcription activator. There are 10 HAP3 homologs in *Arabidopsis* (Kwong et al., 2003). *LEC1-LIKE* (*L1L*) is expressed primarily during seed development. Suppression of *L1L* gene expression induced defects in embryo development that differed from those of *lec1* mutants, suggesting that *LEC1* and *L1L* play unique roles in embryogenesis. *LEC2* encodes

another transcription factor containing a B3 domain, a DNA-binding motif unique to plants (Stone et al., 2001). Transgenic seedlings ectopically expressing *35S::LEC1* and *35S::LEC2* produce somatic embryos on some parts of the plants, indicating both genes establishes a cellular environment sufficient to initiate embryo development.

Another mutant in *Arabidopsis*, *pkl*, like *35S::LEC* plants, initiates somatic embryo development (Ogas et al., 1993). Culture of excised *pkl* roots on hormone-free medium generated somatic embryos; thus *pkl* fails to suppress embryo development in seedling roots. It has been shown that *LEC1* is de-repressed in *pkl* roots. The activation of *LEC1* and possibly also *LEC2* in *pkl* roots initiates the embryonic program and causes somatic embryo development. *PKL* encodes a CHD3 protein (including a chromo domain, a SNF2-related helicase/ATPase domain, and a DNA-binding domain) that has been implicated in chromatin remodeling and repression of transcription (Oags et al., 1999).

Similar to *PKL* and *LEC*s gene, ectopic expression of *BABY BOOM* (*BBM*) triggers a conversion from vegetative to embryonic growth. *BBM* was identified among the genes that are upregulated during the *in vitro* induction of embryo development from immature pollen grains of *Brassica napus* (microspore embryogenesis). *BBM* shows similarity to the *AP2/ERF* family of transcription factors and is expressed preferentially in developing embryos and seeds. Ectopic expression of *BBM* in *Arabidopsis* and *Brassica* led to the spontaneous formation of somatic embryos and cotyledon-like structures on seedlings. The expression pattern of *BBM* in developing seeds combined with the *BBM* overexpression phenotype suggests a role for this gene in promoting cell proliferation and morphogenesis during embryogenesis.

The *Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1* (*AtSERK1*) gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture (Hecht., et al 2001). Ectopic expression of the full-length *AtSERK1* cDNA under the control of the cauliflower mosaic virus 35S promoter did not result in any altered visual plant phenotype. But, seedlings did exhibit a 3- to 4-fold increase in efficiency of initiation of somatic embryogenesis.

Table 1.1 Some embryo mutants identified in *Arabidopsis*.

Mutants	Phenotypes	Gene products
SIN1	Homozygous maternal effect alterations in embryo pattern symmetrical deletion of embryonic axis decreased embryo viability	DICER1, multidomain protein, DEAH box RNA helicase C domain two Ribonuclease III domain SPI/PAZ motif
GNOM	Apical-basal polarity of the embryo lack of root, reduced apical structure	Guanine nucleotide exchange factor for coordination of cell polarity
GURKE	Affects the apical domain. abolishes apical structures in *gk* disorganized green mass of cells at the apical end of *gk* seedlings	Not cloned
CUP1/CUP2	completely fused cotyledons surrounding the apex	NAC-domain proteins
PIN1	cotyledon fusion and dissymmetric organ positioning	a transmembrane protein, catalytic auxin efflux carrier

MP	cotyledon fusion and dissymmetric organ positioning	a member of the *AUXIN RESPONSE FACTOR* (*ARF*) gene family
LECs	specify suspensor cell fate cotyledon identity acquisition of desiccation tolerance suppression of germination trichome on cotyledons in mutants 35S::LECs induce embryo growth in vegetative cells. (Lotan etal., 1998; Stone et al., 2001)	LEC1 is a HAP3 CCAAT box-binding transcription factor LEC2 is a B3 domain transcription factor
PKL	somatic embryo growth on pkl roots on hormone-free media	CHD3 protein, repressing transcription by chromation remodeling
BBM	somatic embryo growth ectopic expression of *BBM*	similarity to the AP2/ERF family of transcription factors

1.2.2 Endosperm development

Recent studies in the eudicot, *Arabidopsis*, and the monocot, Barley, indicate that the basic steps of endosperm development are conserved between monocots and dicots (Olsen et al., 1999; 2001). The endosperm develops from the fertilized central cell. Endosperm development in these species progresses through four phases: syncytial development, cellularisation, differentiation, and death (Fig. 1.4). After fertilization and during early development, nuclear division in the endosperm is not followed by cell wall formation (or cytokinesis), leading to the

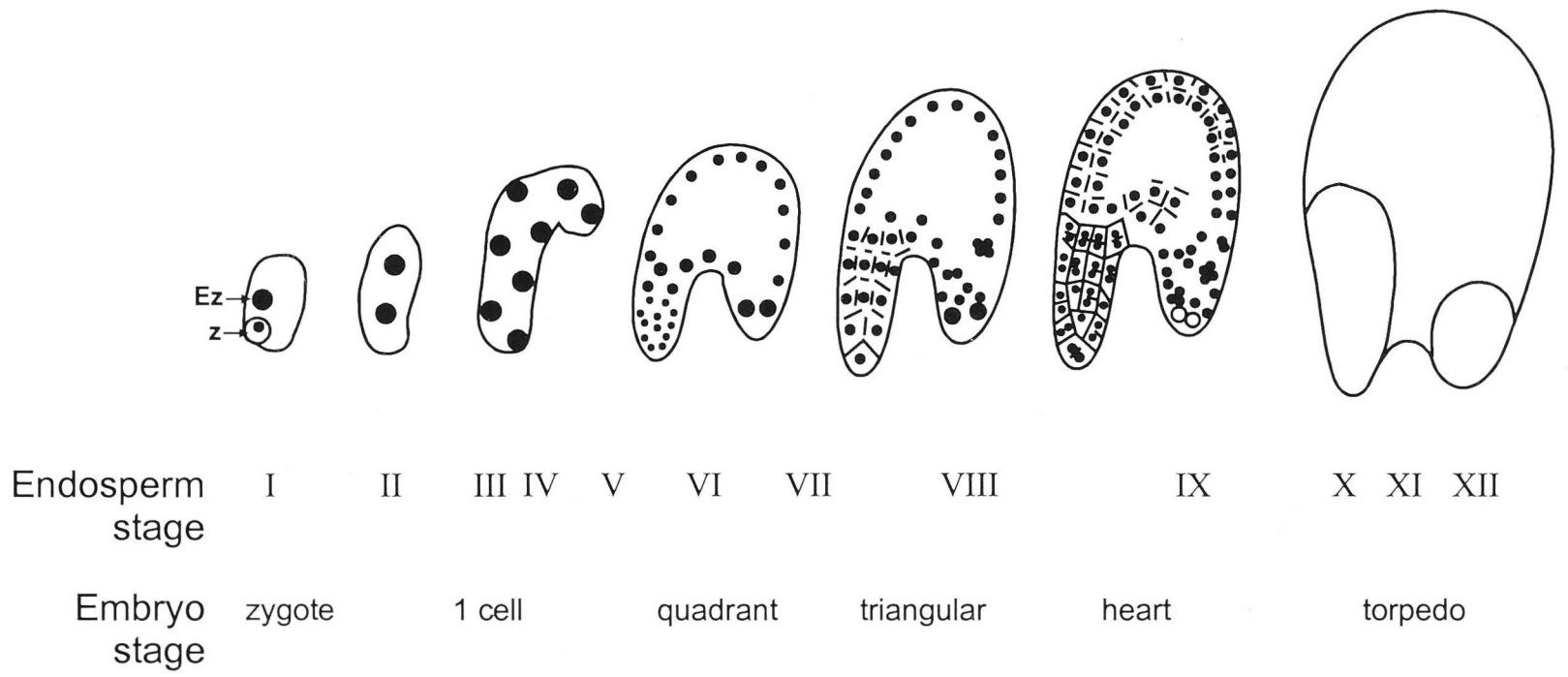


Fig 1.4 Development of the *Arabidopsis* endosperm. Each division of endosperm is associated with the embryo stage. Ez, endosperm zygote; z, zygote. Endosperm cellularisation starts after the 7th division.

development of a syncytium. The *Arabidopsis* endosperm at the syncytial stage is divided into three domains along the major polar axis (Boisnard-Lorig et al., 2001): the micropylar pole occupied by the embryo-surrounding region (ESR), which is densely cytoplasmic; the peripheral endosperm (PEN), which is the largest domain in terms of volume and number of nuclei; the chalazal pole, a pocket which contains few nuclei and constitutes the chalazal endosperm (CZE). The syncytial development of *Arabidopsis* endosperm is divided into successive stages characterized by the total number of nuclei. There are about 200 nuclei at the time of cellularisation. During syncytial development, the formation of nuclear cytoplamic domains (NCDs), cytoplasm in which a nucleus is centered by a radial system of microtubules, is observed in a range of species (Brown et al., 1999).

In endosperm development of angiosperms, the transition from a multinucleate syncytial stage to a cellular stage is marked by formation of the alveoli, a tube-like cell structure. Periclinal divisions in the aveoli lead to the process of cytokinesis and cellularisation of endosperm. Cellularisation begins in the ESR and spreads like waves toward the chalazal region in *Arabidopsis*. Following cellularisation, endosperm cells differentiate into tissue types such as starchy endosperm, and aleurone in cereals. In many eudicot species, endosperm cells are thought to undergo apoptosis: accompanied by an increase in nuclease activity and the internucleosomal degradation of nuclear DNA, hallmarks of apoptosis in animals. Moreover, ethylene and abscisic acid are keys to mediating Programmed Cell Death in cereal endosperm (Young et al., 1997; 2000).

Endosperm has been thought to support embryo development by producing large amounts of storage proteins, starch, and lipids and by

sequestering nutrients from maternal tissue (Lopes and Larkins, 1993). Beyond that, the endosperm is a source of signals involved in embryogenesis (van Hengel et al., 1998) and that there are interactions between the embryo and the endosperm (Opsahl-Ferstad et al., 1997).

1.2.3 Mutations affecting endosperm development

Because endosperm development is complex, mutation in any one of many genes could affect endosperm development. The *titan* (*ttn*) mutants of *Arabidopsis* exhibit striking defects in endosperm development (Tzafrir et al., 2001). The defining feature is the presence of abnormal endosperm with giant polyploid nuclei. Several *TTN* genes encode proteins for structural maintenance of chromosomes (condensins and cohesins) and are required for chromosome function at mitosis. Another *TTN* gene product TTN5 is related to ADP ribosylation factors (ARFs), members of the RAS family of small GTP binding proteins that regulate various cellular functions in eukaryotes. The titan phenotype can therefore result from disruption of chromosome dynamics or microtubule function. Three other genes have been identified that affect endosperm nuclear morphology. *TTN4* and *TTN9* appear to encode plant-specific proteins of unknown function. TTN6 is related to the isopeptidase T class of deubiquitinating enzymes that recycle polyubiquitin chains following protein degradation. Disruption of this gene may reduce the stability of the chromosome complex. Further analysis of the TITAN network should help to elucidate the regulation of microtubule function and chromosome dynamics in seed development.

Rgf1, a mutation reducing grain filling in maize affects basal endosperm and pedicel development (Maitz et al., 2001). Invertase activity is low in

an *rgf1* mutant compared with wild type. The maize (*Zea mays*) *CRINKLY4* (*cr4*) gene encodes a receptor-like kinase that controls a variety of cell differentiation responses, particularly in the leaf epidermis and in the aleurone of the endosperm (Becraft et al., 2001). In the *crinkly4* mutant, the aleurone layer does not form properly.

Sorensen et al. (2002) by analysis of several *Arabidopsis* mutants, such as *knolle*, *pleiade, hinkel*, *runkel* and *open house*, showed that most *Arabidopsis* mutations affecting cytokinesis in the embryo also impair endosperm cellularisation. Mutant *knolle* is a result of the loss of function of a cytokinesis-specific syntaxin gene (Lauber et al., 1997). These results imply that cellularisation and cytokinesis share components of the same basic machinery. They further report the identification of mutations in a novel gene, *SPATZLE*, that specifically interferes with cellularisation of the endosperm, but not with cytokinesis in the embryo. The *spatzle* mutant produced viable seeds in the absense of cellularisation of the endosperm. Analysis of the *spatzle* mutant might identify a specific checkpoint for the onset of cellularisation.

Other studies have led to the identification of a group of important mutants: the *fis* (*fertilization independent seed*) mutants in *Arabidopsis* (Chaudhury et al., 1997; Ohad et al., 1996; Grossniklaus et al., 1998). All the mutants have similar phenotypes: an embryo sac carrying the *fis1/mea* or *fis2* mutations, in the absence of fertilization, develops into a seed like structure with cellularised endosperm and that carrying *fie/fis3* develops a similar structure with syncytial endosperm. The autonomous endosperm formation indicates that *FIS* genes are possible components of apomictic seed development. Following fertilization, the

seeds derived from the mutant embryo sacs arrest with the embryo at heart stage and with the endosperm failing to cellularise. Genetic analysis

Table 1.2 Some *Arabidopsis* endosperm mutants

Mutants	Phenotypes	Gene products
ttn1	enlargement of endosperm nuclei giant cells with enlarged nuclei in embryo	Tubulin-folding cofactor D
ttn2	enlargement of endosperm nuclei embryo with small cells arrested early	Gene identity unknown
ttn3	enlargement of endosperm nuclei embryo viable	SMC2 condensin
ttn6	enlargement of endosperm nuclei embryo arrested at early heart-stage	Deubiquitinating enzyme
ttn5	similar to *ttn1*	ARL2 GTPase
ttn7	similar to *ttn2*	SMC3 cohesin
ttn8	similar to *ttn2*	SMC1 cohesin
ttn9	similar to *ttn2*	Unknown function
knolle	defects of cytokinesis in the embryo and endosperm defect	Syntaxin
spatzle	no endosperm cellularisation cytokinesis normal in embryo embryo normal	not cloned
fis1	no endosperm cellularisation embryo arrested at heart stage autonomous growth endosperm in absent of pollination	Ehancer of zeste like
fis2	similar to *fis1*	Suppresser of zeste 12 like
fis3	similar to *fis1*	Extra sex combs like

indicates that the seed phenotype is determined only by the genotype of the embryo sac irrespective of the genotype of the sperm. So these mutations are female-gametophytically controlled and the defect of the mutant ovule cannot be rescued by wild type pollen. Thus the reciprocal crosses between *fis* mutants and wild type display distinct results, suggesting the effect of parent-of-origin and the different contributions of paternal and maternal copies of *FIS* genes.

The predicted protein sequences encoded by these three genes show that MEA/FIS1, FIE/FIS3 and FIS2 are related to the polycomb group proteins, Enhancer of zeste, Extra sex combs and Suppresser of zeste 12 previously described in *Drosophila* (Luo et al., 1999; Grossniklaus et al., 1998; Ohad et al., 1999; Chaudhury et al., 2001). Similar genes have been isolated in other organisms. FIS1/MEA and FIE/FIS3 proteins physically interact with each other to form a protein complex, and possibly with other proteins, as do the polycomb group counterparts in other species (Luo et al., 2000; Spillane et al., 2000). The polycomb group protein complex possibly represses target gene expression by preventing access of transcription factors by remodeling the chromatin structure. FIS2 also show homology to EMBRYONIC FLOWER2 (EMF2), (Yoshida et al., 2000), and VERNALIZATION 2 (VRN2) (Gendall et al., 2001) in *Arabidopsis*.

Apart from the role in repressing endosperm cell division without fertilization, *FIS* genes appear to be potent regulators of the establishment of the anterior-posterior polar axis in the endosperm (Sorensen et al., 2001). When *fis* ovules are fertilized with *FIS* pollen, chalazal cysts are produced not only in the chalazal region but also elsewhere and

endosperm markers normally expressed in the chalazal region are also expressed in other endosperm domains (Sorensen et al., 2001).

FIE-mediated polycomb complexes are also an essential component of a floral repression mechanism established early during plant development (Kinoshita et al., 2001). Mutations in the *FIE* polycomb gene caused the seedling shoot to produce flower-like structures and organs. Flower-like structures were also generated from the hypocotyl and root, organs not associated with reproduction. Expression of floral induction and homeotic genes was de-repressed in mutant embryos and seedlings.

1.3 Parent-of-origin effects on seed development

1.3.1 Reciprocal interploidy crosses

Seed developing from interploidy crosses often show different seed phenotypes depending on which parent was the female or male. The endosperm often shows more dramatic change than the embryo (Brink and cooper, 1947; Maheshwari, 1950; Bhatnagar and Sawhney, 1981; Vijayaraghavan and Prabhakar, 1984; Lopes and Larkins, 1993). The dramatic endosperm change in interploidy crosses and the loss of the nourishing role to the embryo suggest that endosperm failure is the primary cause of seed abortion (Johnston et al, 1980; Kermicle and Alleman, 1990; Haig and Westoby, 1991; Birchler, 1993).

A variety of conflicting hypotheses have been offered to explain the data from interploidy crossing studies (revieved by Haig and Westoby, 1991). The hypotheses were carefully examined by Lin (1984) through a series of crosses in maize, using 2n or 4n pollen parents, and 2n seed parents

that contributed varying numbers of polar nuclei to the endosperm due to the indeterminate gametophyte mutation. Thus Lin generated seeds with 2n sporophytic maternal tissues and either 2n or 3n embryos, but with a range of endosperm that varied both in total ploidy and in the balance of maternal to paternal genomes. The conclusion is that a 2maternal:1paternal genomic ratio rather than a specific ploidy level is the critical factor for normal endosperm development. Lin (1984) interpreted the 2m:1p ratio requirement as an indication that the paternally imprinted genes are involved in endosperm development. Thus the contribution to endosperm development between the maternal and paternal genomes is not functionally equivalent. Scott et al. (1998) presented a cytological investigation of seed development following interploidy crosses in *Arabidopsis*. Crosses between diploid and tetraploid plants in either direction, resulting in double the normal dose of maternal or paternal genomes in the seed, produce viable seeds containing triploid embryos. However, development of the seed and in particular the endosperm is abnormal, with maternal and paternal genomic excess producing complementary phenotypes. A double dose of maternal genomes with respect to the paternal contribution inhibits endosperm development and ultimately produces a smaller embryo. In contrast, a double dose of paternal genome promotes growth of the endosperm and embryo. Reciprocal crosses between diploids and hexaploids, resulting in a triple dose of maternal or paternal genomes, produce seeds that begin development with similar but more extreme phenotypes than those with a double dose, but these invariably abort. One explanation of the observations is that seeds with maternal or paternal excess contain different doses of maternally or paternally expressed imprinted loci affecting endosperm development. This hypothesis is illustrated in Fig. 1.5.

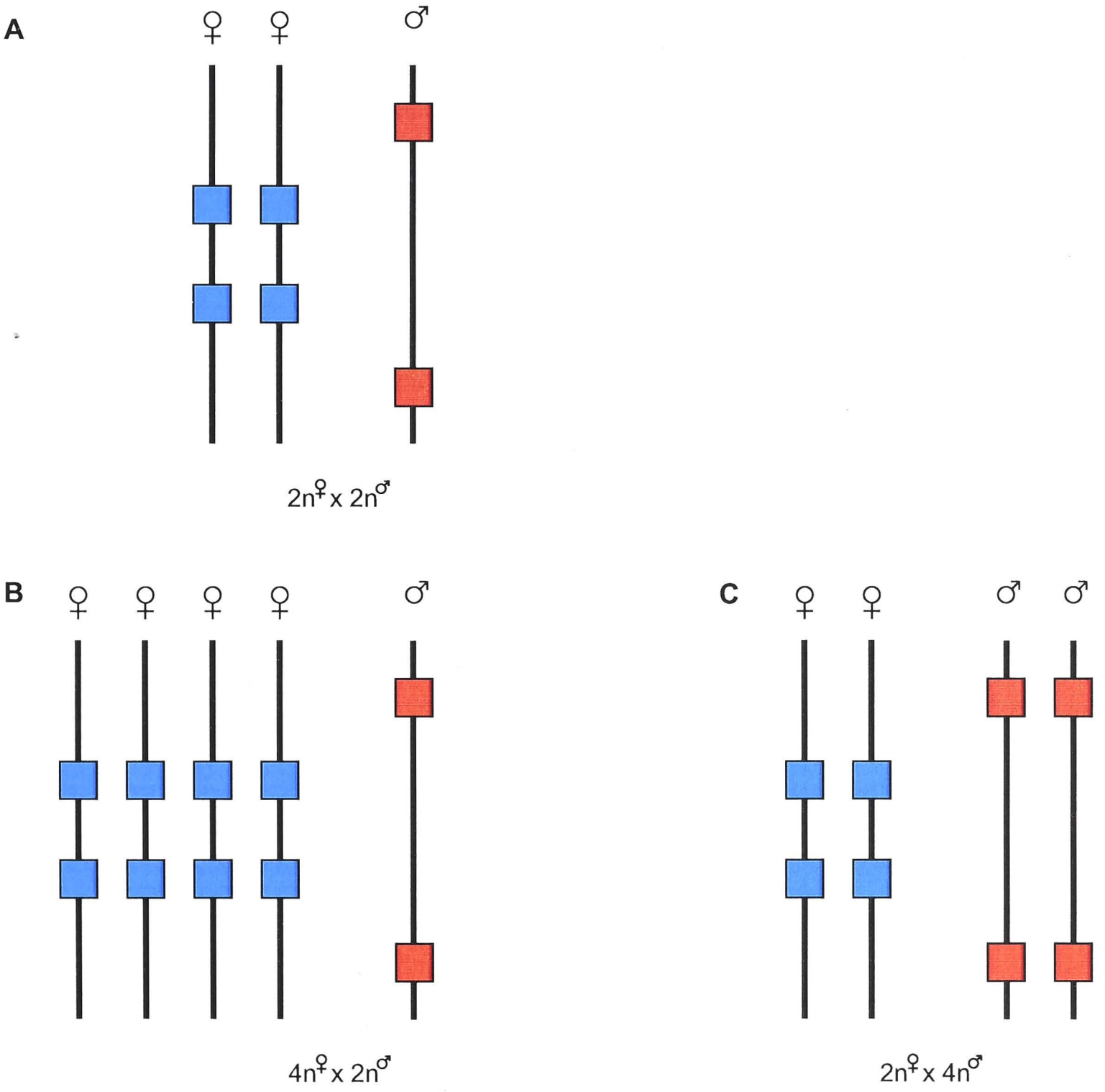


Fig. 1.5 Schematic illustration of the parental conflict theory. (A) Triploid endosperm of a diploid pollinated (2n) with a diploid (2n), with maternal and paternal balance of 2m:1p . (B) Endosperm from a tetraploid (4n) pollinated with a diploid, with maternal excess of 4m:1p . Endosperm development is reduced. (C) Endosperm from a diploid (2n) pollinated with a tetraploid (4n), with paternal excess of 2m:2p . Endosperm development is promoted. Blue square, active maternal copy of a gene. The paternal copy of this gene is silenced (imprinted). These genes repress endosperm development; Red square, active paternal copy of a gene. The maternal copy of this gene is silenced (imprinted). These genes promote endosperm development.

The parental conflict theory attempts to explain the genomic imprinting in flowering plants and mammals (Haig and Westoby, 1989; 1991). A mother may have offspring by more than one father. Embryos require a significant amount of resources from maternal tissues. As a result, fathers (or the paternal genome) strive to extract the maximal amount of resources for their own offspring, whereas the mother (or the maternal genome) endeavors to allocate equal amounts of resources among offspring. During evolution, imprinting arose from the conflict between maternal and paternal genomes. The battle field would be the nutrient acquisitive tissues: the endosperm in plants and placenta in mammals. Genes tending to increase nutrient flow to the embryo and promote endosperm growth would be preferentially expressed by the paternal alleles, and the maternal allele would be silenced. By contrast, genes tending to restrict nutrient flow to the embryo and suppress endosperm growth would be preferentially expressed by the maternal alleles, and the paternal allele would be silenced. It is clear that Scott et al.'s (1998) observation is consistent with the parental conflict theory. When an *Arabidopsis* tetraploid was pollinated with a diploid, a double dose of maternal genomes with respect to the paternal contribution inhibits endosperm development and ultimately produces a smaller embryo. In contrast, when the diploid was pollinated with a tetraploid, a double dose of paternal genome from the sperm promotes growth of the endosperm and embryo.

1.3.2 DNA Methylation, gene imprinting and seed development

DNA methylation plays an essential role in regulating plant development. Genome wide demethylation has a pleiotropic effect on plant

morphology, including homeotic transformations in floral organs and altered flowering time (reviewed by Finnegan et al., 2000). Methylation generally represses transcription either directly, by blocking the binding of transcription factors, or indirectly, through proteins that bind methylated DNA resulting in deactylation of nearby histones and decreased transcription (reviewed by Ng and Bird, 1999). All may be due to the change in chromatin caused by methylation of DNA.

Gene imprinting is a mechanism by which the expression states, after fertilization, of a set of gene copies are dependent on the parental origin. This mechanism involves an epigenetic marking of a certain set of alleles in the germline, maintenance of the mark through cell division, and response to the mark resulting in uniparental gene expression after fertilization. Therefore, each genome contributes a different set of active alleles to the offspring, which develop abnormally if the parental genome balance is disturbed. DNA methylation has been shown to be related to gene imprinting in mice (Sanford et al., 1987). Gene imprinting is essential for embryo development in mice. Although in the mouse most imprinted genes show allelic differences in DNA methylation, it is not clear whether methylation regulates the expression of some or all imprinted genes in somatic cells. To examine the mechanisms of silencing of imprinted alleles, (El Kharroubi et al., 2001) generated novel uniparental mouse embryonic fibroblasts containing exclusively either the paternal or the maternal genome. These fibroblasts retain parent-of-origin allele-specific expression of 12 imprinted genes examined for more than 30 cell generations. They demonstrated that changes in DNA methylation but not histone acetylation created a heritable epigenetic state at some imprinted loci (such as H19) in somatic cells.

In plants, the results of interploidy crosses show that imprinting of genes is involved in endosperm development (Lin et al., 1984; Scott et al., 1998). Because ovules carrying a *fis* mutant gene gives rise to mutant seeds, irrespective of the status of the paternal *FIS* genes, the effect of parent-of-origin and the nonequivalent contribution of paternal and maternal copies of *FIS* genes is indicated. The *FIS* genes may be imprinted during endosperm development and controlled by DNA methylation. In other words, DNA-methylation may play a role in seed development by mediating parent-of-origin effects. This hypothesis will be thoroughly investigated in this PhD study.

In mice and humans, *H19* is expressed almost exclusively from the maternally inherited chromosome, while *Igf2* expression is mostly from the paternal chromosome (Kaffer et al., 2001). A recent study suggested that the paternal genome is silenced during the early seed development in *Arabidopsis* (Vielle-Calzada et al., 2000). None of the paternally derived alleles of 20 loci, involved in various processes and distributed throughout the genome, was expressed during early seed development in *Arabidopsis*, indicating that most, if not all, of the paternal genome may be initially silenced. Thus, contrary to previous interpretations, early embryo and endosperm development are mainly under maternal control. However, this conclusion was questioned by Weijers et al. (2001). They found that during early embryogenesis in *Arabidopsis*, a paternal copy of a chimaeric gene, AtRPS5A::GUS was expressed as early as at the two cell embryo stage. Their findings indicate that there is no overall maternal control of early embryogenesis, and that the contribution of the parental alleles needs to be assessed for each gene individually. This observation challenges the conclusion made by Vielle-Calzada et al. (2000). The

paternal copies may not be totally silenced for some important genes involved in early embryo and endosperm development.

1.4 Apomixis

1.4.1 What is apomixis?

Some higher plants reproduce asexually by apomixis, a natural way of cloning via seeds. Apomictic plants produce progeny that are an exact genetic replica of the mother plant. In the apomictic species, the embryo is derived solely from the unreduced egg cell (Gametophytic apomixis) or the somatic tissue (Adventitious embryony) without the egg and sperm fusing (reviewed by Koltunow, 2001). The origin of the endosperm supplying the apomictic embryo is more complicated: some apomicts are able to produce endosperm autonomously and some are not, that is they need fertilization of the central cell nuclei to produce endosperm (Pseudogamy). There are three apomictic mechanisms; diplospory, apospory and adventitious embryony. In diplospory, the megaspore mother cell switches from a sexual to an apomictic pathway to produce an unreduced embryo sac and an autonomous embryo is produced in this type of apomixis with endosperm derived autonomously or sexually (fertilization of the polar nucleus is necessary). In apospory, the somatic cells in the nucellus form unreduced embryo sacs and the embryos are derived from the unfertilized egg cell while the endosperm formation can be autonomous or result from fertilization. In adventitious embryony, embryos develop from cells in somatic tissues external to the sexual embryo sac and the endosperm has a sexual origin.

In some apomicts, diplospory can occur as late as the second meiotic division.

1.4.2 Components and genetics of apomixis

In gametophytic apomixis, the successful development of an asexual embryo includes three basic developmental components: apomeiosis (the formation of unreduced embryo sac) by apospory or diplospory, parthenogenesis of the embryo, and endosperm formation either sexually or asexually. The information on the genetic control of apospory in *Pennisetum*, *Panicum*, and *Ranunculus* suggests that apospory is probably controlled by a single dominant locus (Asker and Jerling, 1992). Recent reports in dicotyledonous plants, *Erigeron annus* (Noyes and Rieseberg, 2000) and *Taraxacum officinale*, (Van Dijk et al., 1999) however, have shown that in some species apomeiosis and parthenogenesis can segregate independently, suggesting that they probably rely on different genes. Attempts to map apomixis in three related forage grass species, including *Tripsacum* (Grimanelli D etal., 1998), *Brachiaria* (Pessino et al., 1997) and *Paspalum* (Labombarda et al., 2002) have shown that the genomic regions that control apomixis are distinct, suggesting that apomixis arose through the action of different genes. Thus apomixis may be controlled by a complex gene set. In some species, one major gene is enough to trigger a cascade of the apomoxis pathway. However, in other apomicts several genes are essential to master different components of apomixis. In *Arabidosis*, there are no natural apomicts. However, the autonomous development of endosperm in *fis* class mutants support the idea that different components of apomixis rely on different genes and apomixis may be dissected genetically (Chaudhury et al., 1997).

1.4.3 Apomixis and genomic dosage

In many plants, genomic dosage is critical for endosperm development. Lin et al. (1984) showed that normal endosperm development requires a

maternal to paternal genome ratio of 2m:1p; deviations lead to seed abortion. *Arabidopsis* is less susceptible to changes in the 2m:1p ratio (Scott et al., 1998). But any variation from the 2m:1p ratio still causes a profound change of seed phenotype. The requirement for a 2m:1p ratio was interpreted as genomic imprinting (Lin et al., 1984). In a sexual plant, the endosperm needs the fertilization of the central cell to develop. It has been postulated that the central cell nuclei are repressed by the *FIS* genes (Chaudhury et al., 1999). The removal of *FIS* function by mutation will trigger autonomous endosperm development from an unfertilized central cell following a similar pattern in the wild type. Some apomicts evolved to bypass the requirement of balanced expression of imprinted genes for autonomous endosperm development. The removal of the *FIS* gene function in those autonomous apomicts may be essential for autonomous endosperm development.

Most

~~There are a few~~ apomicts ~~that~~ need fertilization of the central cell nuclei for proper endosperm development. But how does the maternal and paternal genome ratio affect the endosperm development in apomicts with unreduced embryo sacs? *Panicum* and many aporsporous plants have a modified embryo sac, of which the central cell only contains a single unreduced nucleus. Thus the fertilization of the central cell produces a 2m:1p balanced endosperm (Grimanelli et al., 2001). However, apomictic *Tripsacum* is apparently tolerant to deviations from 2m:1p dosage effects. The endosperm of apomictic *Tripsacum* develops normally with 2m:1p, 4m:1p, 4m:2p, 8m:1p and 8m:2p (Grimanelli et al., 1997). Thus, specific dosage effects are seemingly not required for endosperm development in this species. These findings suggest that evolution of diplosporous apomixis might have been restricted to species with few or no imprinting requirements, and the findings have strong implications regarding the

transfer of apomixis to sexually reproducing crops with strict requirement of a 2m:1p ratio. Identification of those imprinted genes mediating the dosage response will elucidate the molecular basis of endosperm development.

1.5 Endosperm development and seed size

The intrinsic size of plant organs is determined by developmental signals and environmental factors, yet the molecular and genetic mechanisms that control organ size are largely unknown. The size of each organ is associated with the ability of individual plants to compete against other species or its own siblings. Functional analysis of *Arabidopsis* genes is defining important regulators involved in these mechanisms. *AINTEGUMENTA* (*ANT*) was previously shown to be involved in floral organ initiation and growth in *Arabidopsis*. *ant* flowers have fewer and smaller floral organs and possess ovules that lack integuments and a functional embryo sac due to decreased cell division within organ primordia. Ectopic expression of *ANT* under the control of the constitutive 35S promoter results in the development of larger floral organs due to increased cell division and cell expansion. In addition, *35S::ANT* ovules often exhibit increased growth of the nucellus and the funiculus. These results suggest that *ANT* stimulates cell growth in floral organs (Krizek et al., 1999) by regulating cell proliferation and organ growth through maintaining the meristematic competence of cells during organogenesis (Mizukami and Fischer, 2000).

Seed is an important organ of plants determining the success of offspring in an ecosystem. The number and size of viable seeds produced by different species have been considered important adaptive characters

(Harper et al., 1970.). Often there is a negative correlation (tradeoffs) found between seed size and seed number. This is due to the limited resources of the maternal supply. How a successfully evolved species maintains the balance between the number and size of seed it produces are not known. However under artificial selection, both seed size and seed number can be significantly increased for a cultivar compared with a wild type counterpart. Seed size is an important yield factor for agricultural use of plants. However, information about the molecular and genetic basis of control of seed size is very limited.

Generally, the genetic analysis of seed size is confined to quantitative genetic methodology. High heritabilities have been reported for crop species grown under controlled experimental conditions, in contrast to the lower values often found in the wild (Silvertown, 1989). The analysis of reciprocal crosses in several species have generally shown strong maternal effects, indicating that seed size is influenced by the phenotype of the mother plant, by the maternal genotype of the seeds, and/or by the interactions between the mother plant and the offspring (Roach & Wulff, 1987). QTL mapping studies allow the identification of genomic regions controlling a quantitative trait. In addition, the analysis of multiple traits in the same experimental mapping population enables the detection of loci with pleiotropic effects on various characters (Prioul et al, 1997). Several seed size QTL analyses have been performed in domesticated species (Lu et al, 1997, Maughan et al, 1996, Paterson et al, 1995). Alonso-Blanco et al (1999) analyzed two *Arabidopsis* strains differing in the mean seed size and seed number they produced. Maternal and nonmaternal genetic factors were involved in the seed size variation, and interactions between both types of factors presumably occurred. They mapped quantitative trait

loci (QTLs) affecting 12 life history traits related to seed size, fruit size, seed number, and plant resources.

Polyploidization in plants directly influences organ size including seed size. Many crop products, such as cotton, wheat, and banana, have high-ploidy varieties, which produce larger flowers, grains, or fruits than those of diploid counterparts. Possibly the polyploidy changes the expression of some genes and causes organ size changes. Lee and Chen (2001) reported ploidy-dependent, epigenetically altered gene expression among diploid *A.thaliana*, diploid *Cardaminosis arenosa* and allotetraploid *Arabidopsis suecica*, each of which has quite different organ sizes.

There are some metabolic genes involved in seed development. Seed-specific over-expression of an *Arabidopsis* cDNA encoding a diacylglycerol acyltransferase enhances seed oil content and seed weight (Jako et al., 2001). In maize, an endosperm-specific gene *shrunken2* (*Sh2*) encodes the large subunit of the heterotetrameric starch synthetic enzyme adenosine diphosphoglucose pyrophosphorylase (AGP)(Giroux et al., 1996). Modifying AGP increased seed weight 11-18% without increasing or decreasing the percentage of starch. These results show that single gene mutations giving rise to increased seed weight, are clearly possible in a plant with a long history of intensive and successful breeding efforts. The same gene functioned in wheat to increase the grain weight (Smidansky et al, 2002). Therefore, the allosteric enzyme ADP-glucose pyrophosphorylase (AGP) plays a key role in regulating starch biosynthesis in cereal seeds and is likely to be an important determinant of seed sink strength.

In *Arabidopsis* seed size is subject to parent-of-origin effect. As reviewed in section 1.4.1, the reciprocal crosses between diploid and tetraploid plants produce seeds of different sizes (Scott et al, 1998). The higher paternal genome dosage promotes endosperm proliferation and causes bigger seed size, while the high maternal genome dosage restricts seed growth.

1.6 This study

The aim of this project is to discover genes with functions in early seed development, using *Arabidopsis* as the experimental material. The early seed development is defined here to be the stage spanning fertilization to the embryo heart stage when the endosperm has almost cellularised.

In Chapter 2, I focus on the *FIS* class genes controlling early stages of endosperm development by investigating how the parent-of-origin or imprinting displayed by these genes affects seed development. When I started this PhD project, the *fis* class mutants had been well characterized and the corresponding genes cloned. There was little information about the expression pattern of these genes and how it might relate to gene function. I have used the *GUS* reporter gene system to investigate the expression pattern of the *FIS* class genes. The promoters and part of coding sequence of *FIS* class genes were fused to a *GUS* reporter gene and the transgenic plants were studied for the *GUS* expression pattern and how this was affected if the transgene was delivered via the egg or the pollen. The expression profiles of *FIS* genes and their paternal imprinting are described in this chapter and also published (Luo et al., 2000).

In chapter 3, the effect of changes of DNA methylation on the *fis* mutant seeds is investigated. DNA methylation has been described as controlling gene imprinting in the animal kingdom. However little is known about its roles in plant gene imprinting. Expression of the *FIS* genes and the effect on imprinting were examined under hypomethylation conditions using transgenic plants containing an antisense construct of a DNA methyltransferase gene. Seed development of the *fis* mutants and the wild type was also studied under hypomethylation conditions, leading to the discovery of a new role for DNA methylation in seed development. In this chapter, I demonstrate that the maternal defect of each of the *fis* class mutants can be rescued by pollen from low methylation parents. Seed size was reduced if a wild type plant was pollinated with the hypomethylated pollen. The rescue of *mea/fis1* and *fis2* defect did not involve the functioning or reactivation of paternally imprinted *MEA/FIS1* and *FIS2*, indicating that imprinting of the paternal *FIS1/MEA* or *FIS2* is not controlled by DNA methylation. The *fis2* and *mea/fis1* maternal defect could be rescued by a hypomethylated paternal genome not by a hypomethylated maternal genome. I mapped three putative paternal modifiers for *fis* genes, which were possibly regulated by DNA methylation.

In chapter 4, I describe a new screening experiment for mutants that produced seeds with small size. The purpose of this screening was to identify genes that control endosperm development and are also regulated by DNA methylation. Given the fact that wild type ovules give small seeds, when pollinated with hypomethylated pollen, I postulated that some genes controlling seed size could be regulated by DNA methylation. In this chapter, two mutants with reduced seed size were characterized cytologically and genetically. I cloned one gene that was responsible for

the seed size change. This gene is a member of the plant *WRKY* transcription factor family. The role of this gene in endosperm development and its relationship to hypomethylation have been investigated.

CHAPTER 2

The expression pattern and imprinting of *FIS* class genes in *Arabidopsis*

2.1 Introduction

Arabidopsis seed, like the seed of other angiosperms, is a product of double fertilization, in which one of the two sperm cells fertilizes the haploid egg cell, giving rise to a diploid embryo, and the other sperm cell fertilizes the polar nuclei in the central cell, giving rise to the triploid endosperm (reviewed by Chaudhury et al., 2001). As the embryo and the endosperm develop, the ovule enlarges into a seed; the maternal tissues of the inner and outer integuments surrounding the embryo sac form the seed coat. The egg cell and the central cells of the early embryo sac do not normally show any development in the absence of pollination and fertilization. However, in many families of plants, seed development can occur without fertilization of these cells (autonomous apomixis) or where only fertilization of the central cell occurs (pseudogamous apomixis).

Peacock et al. (1995) and Chaudhury et al. (1997) have proposed a strategy for identifying genes that uncouple components of seed development from the fertilization process. For the isolation of these mutants, Chaudhury et al. (1997) used the stamenless mutant, *pistillata* (*pi*) (Goto and Meyerowitz, 1994). If *pi* plants are not pollinated, the siliques remain short; they only elongate when seed is formed. They identified three mutants in which the siliques elongated without pollination and formed seed-like structures, called *fis1* (*f*ertilization-*i*ndependent *s*eed), *fis2*, and *fis3*. At approximately the same time Ohad

et al. (1997) described a mutant that forms endosperm without fertilization, called *fie* (*fertilisation independent endosperm*). Another mutant, *medea* was first described as a gametophytic maternal effect mutant, in which the embryos derived from *mea* eggs abort irrespective of the paternal contribution (Grossniklaus et al., 1999). Later Luo et al. (1999) demonstrated that *mea* was an allele of *fis1*, and *fie* was an allele of *fis3*.

In the *fis* mutants when fertilization is prevented by the removal of pollen-producing anthers, the mutant central cell replicates to form a diploid endosperm, and the maternal ovary and ovule integuments generate the fruit and seed coat, respectively. Thus the *FIS* genes are repressors of seed development. When *FIS* genes are mutated seed development initiates. The autonomous endosperm of *fis1/mea* and *fis2* develops in a similar way to wild type fertilised endosperm and cellularises. In the *fis3/fie* autonomous endosperm, the endosperm does not cellularise and remains at the syncycial stage. When homozygous *fis1/fis1*, *fis2/fis2* plants or heterozygous *FIS3/fis3* plants were pollinated with either wild type pollen or the corresponding *fis* mutant pollen, each of the *fis* seeds had the embryo arrested at the torpedo stage, indicating that the *fis* mutation exerts a gametophytic maternal effect on seed development. The heterozygotes of the *fis* class mutants display 50% seed abortion when pollinated. The endosperm derived from the ovules carrying the *fis* gens is over-proliferated and uncellularised (Kinoshita et al., 1999; Sorensen et al., 2001). *FIS* genes also regulate the polarity of the developing endosperm post fertilisation. Sorensen et al. (2001) reported that when *fis* ovules were fertilized, the endosperm patterning along the major polar axis is perturbed. Posterior structures develop in more anterior domains of the endosperm. This correlates with the ectopic

expression of a posterior molecular marker. Thus, *FIS* genes appear to be potent regulators of the establishment of the anterior-posterior polar axis in the endosperm.

The three *FIS* genes have been cloned. *FIS1/MEA* and *FIS3/FIE* are related in sequence to two classes of *Drosophila* polycomb genes, *Enhancer of zeste* (*E(Z)*) and *Extra sex combs* (*ESC*), respectively, which initially were identified to repress homeotic genes (Gutjahr et al., 1995; Jones et al., 1993). Similar genes have been identified in invertebrates, vertebrates and plants, which are also repressors (Hobert et al., 1995; Schumacher et al., 1998).

To maintain cell identity during development and differentiation, mechanisms of cellular memory have evolved that preserve transcription patterns in an epigenetic manner. The proteins of the Polycomb group (PcG) are part of such a mechanism, maintaining genes in a silenced state. They act as repressive multi-protein complexes that may keep target genes inaccessible to the transcriptional machinery, inhibit chromatin remodelling, influence chromosome domain topology and recruit histone deacetylases (HDACs). Depletion of PcG proteins by double-stranded RNA interference leads to de-repression of developmentally regulated genes (Breiling et al., 2001).

Polycomb proteins are thought to alter the chromatin structure of target genes (Fitzgerald and Bender, 2001). Jones et al. 1996 showed that the *Drosophila* ESC and E(z) proteins are direct partners in polycomb group-mediated repression. Physical interaction of the FIS1/MEA and FIS3/FIE in *Arabidopsis* was also demonstrated with a yeast two-hybrid system (Luo et al., 2000; Spillane et al., 2000). FIS2 is likely to be a zinc finger

DNA-binding protein (Luo et al., 1999). A role for FIS2 in chromatin remodelling may be inferred from its homology to the *Drosophila* Polycomb group protein Suppressor of Zeste 12, Su(z)12 (Birve et al., 2001). In *Drosophila*, *Su(z)12* together with other polycomb group repressors, controls *HOX* gene expression. *Su(z)12* mutants exhibit very strong homeotic transformations and *Su(z)12* function is required throughout development to maintain the repressed state of *HOX* genes. Unlike most other PcG mutations, *Su(z)12* mutations are strong suppressors of position-effect variegation, suggesting that *Su(z)12* also functions in heterochromatin-mediated repression. Furthermore, *Su(z)12* function is required for germ cell development. The Su(z)12 protein is highly conserved in vertebrates. In *Arabidopsis* it is related to proteins FIS2, EMBRYONIC FLOWER2 (EMF2), and VERNALIZATION2 (VRN2). *EMF2* is a repressor of floral homeotic genes. Loss-of-function mutations in the *EMF* genes (*EMF1* and *EMF2*) cause *Arabidopsis* to flower directly, bypassing vegetative shoot growth (Yoshida et al., 2001). The *VRN2* gene is involved in vernalization. In wild-type *Arabidopsis*, vernalization results in the stable reduction of the levels of the floral repressor *FLC*. In *vrn2* mutants, *FLC* expression is downregulated normally in response to vernalization, but instead of remaining low, *FLC* mRNA levels increase when plants are returned to normal temperatures. Therefore *VRN2* stably maintains *FLC* repression after a cold treatment, serving as a mechanism for the cellular memory of vernalization.

Because mutation in each of the *FIS* class genes results in similar phenotypes, it is likely that all three genes participate in a polycomb-like complex to control genes involved in seed development, particularly endosperm development. In understanding the roles of these genes, it is crucial to know in which tissues and cells these loci are active and to

determine whether all three genes are active in the same cell types and at the same developmental times.

There were some data describing the tissue-specific expression of *MEA/FIS1* and *FIE/FIS3* (Vielle-Calzada et al., 1999; Ohad et al., 1999). *In situ* hybridization studies have shown that *MEA/FIS1* mRNA is present, before fertilization, in the eight-nucleate embryo sac, in the egg cell, and in the central cell. After fertilization, *MEA/FIS1* mRNA was detected in all cells of the suspensor and the embryo until the heart and torpedo stage of embryo development, thereafter becoming weaker. The *MEA/FIS1* mRNA was also located in free endosperm nuclei but absent from cellularised endosperm. *FIE*, on the other hand, is expressed in the sporophytic and gametophytic tissues: cauline leaf, stem, root, immature floral buds, open flower, and young siliques, as shown by reverse transcriptase-PCR of RNA pools derived from various tissues (Ohad et al., 1999).

MEA/FIS1 has been shown to be imprinted; during early seed development only the maternal allele is expressed. Vielle-Calzada et al. (1999) detected that the triploid endosperm nuclei showed expression of only the two maternal copies of *MEA/FIS1*, suggesting that the paternal copy was silenced. They confirmed the silencing of the paternal *MEA/FIS1* copy in 54 hour old seeds. Kinoshita et al. (1999) showed similar results for the imprinting of *MEA/FIS1*. They found that only the maternal *MEA/FIS1* mRNA was detected in the endosperm from seeds at the torpedo stage and later. However both maternal and paternal *MEA/FIS1* alleles were expressed in the torpedo stage embryo and in other sporophytic tissues such as leaf, stem, and root. Although there are some discrepancies in the description of the expression pattern of

MEA/FIS1 between these two groups; in general, these expression data fit with earlier genetic observations in which reciprocal crosses involving wild-type and mutant alleles of the three *FIS* genes did not give identical results, indicating a parent-of-origin effect (Chaudhury et al., 1997; Ohad et al., 1997; Grossliknaus et al., 1998).

This difference in pattern of expression in early seed development between the paternally and maternally derived genes is similar to the control of imprinted genes reported in a number of cases in the animal kingdom (reviewed by John and Surani, 1996). The *mas* oncogene, originally identified because of its tumorigenic potential, was maternally repressed and p*57kip2*, which encodes a negative regulator of cell proliferation is paternally repressed in both human and mouse. In mice and human, *H19* is expressed almost exclusively from the maternally inherited chromosome, while *Igf2* expression is mostly from the paternal chromosome (Kaffer et al., 2001). More and more imprinted genes have been identified in the animal kingdom and there is a tendency for imprinted genes to exist in chromosomal clusters (Cleary et al., 2001). The importance of gene clustering for the proper regulation of imprinted genes is still an open question (Cleary et al., 2001).

To investigate gene expression, various approaches can be utilised. The *Escherichia coli* beta-*glucuronidase* gene (*GUS*) as a gene fusion marker for analysis of gene expression in transformed plants has been widely used (Jefferson, 1989). Plants expressing *GUS* are normal, healthy and fertile. The GUS product is very stable in plant and can be easily detected with the histochemical substrate x-Glu or through fluorometric assays. In this chapter I report an analysis of the activity patterns of *GUS* reporter

gene constructs of all three *FIS* genes and show differential parent-of-origin activity for each locus.

2.2 Materials and methods

2.2.1 *MEA/FIS1*, *FIS2*, and *FIE/FIS3* promoter::*GUS* fusion constructs

The *MEA*::*GUS* translational fusion contains 2,070 bp of nucleotide sequence upstream of the predicted translational start site, exons 1-15, the first 13 bp of exon 16, and introns 1-15 in the vector pBI101-3 (CLONTECH). *MEA* genomic fragments were excised from a bacterial artificial chromosome (BAC) clone (IGF 10O11). To make this *MEA*::*GUS* promoter fusion, a 3.1 kb *Eco* RI-*Pst* I fragment containing the *MEA/FIS1* promoter region, exon1, 2 , and part of exon 3 was first subcloned from the BAC in to vector pBluescript KS+. A unique *Cla* I site found in intron 2 was used in conjunction with a Not I site in the vector to insert a *Cla* I-*Not* I fragment from vector pART 7 which contains the OCS terminator (Gleave, 1992), called pBlueFIS1short. Then, a 3.26 kb *Cla* I fragment of *MEA/FIS1* spaning from intron2 to exon 16 was isolated from the same BAC and inserted in the correct orientation in the unique *Cla* I site of pBlueFIS1short. From this resulting construct in pBluescript KS+, the 5.8 kb *Bam* HI *MEA/FIS1* fragment was excised and cloned into pBI101-3 to produce the final *FIS1*::*GUS* promoter translational fusion construct.

To make the *FIS2*::*GUS* fusion, a 1.6 kb *Dra* I fragment of the *FIS2* genomic sequence was first excised from the E2 fragment (Luo et al., 1999) and cloned into a *Sma* I site of PBI101-3 vector in a correct

orientation, called PBI101 *Dra* I. A *Hind* III site is within this *Dra* I fragment and another *Hind* III is in the PBI101-3 vector. This *Hind* III fragment was replaced with a longer *Hind* III fragment from the Cosmid 18H1 (Luo et al., 1999) to obtain the final *FIS2*::*GUS* construct. The *FIS2*::*GUS* fusion contains 3,189 bp of nucleotide sequence upstream of the predicted translational start site; exons 1, 2, and 3; the first 39 bp of exon 4; and the first three introns in the vector pBI101-3.

The 11.6 kb *Eco* RI *FIE/FIS3* genomic DNA fragment was excised from the BAC IGF 10A3 and cloned into pBluescript in the correct orientation, so a *Bam* HI fragment can be excised to contain a full length of *FIE* promoter and partial coding sequence, because a *Bam* HI site is found in the promoter region of *FIE/FIS3* and another *Bam* HI site is located on vector. This *Bam* HI fragment was then cloned in the vector pBI101-3 (CLONTECH) to obtain a *GUS* fusion. The *FIE*::*GUS* fusion contains 6,970 bp of *FIE/FIS3* promoter sequence upstream of the predicted start codon, the first eight exons and eight introns of genomic sequence, and 50 bp of the ninth exon in the vector pBI101-3 (CLONTECH).

All binary constructs were mobilized in *Agrobacterium tumefaciens* AGL1, and the T-DNA was introduced into *Arabidopsis* ecotypes WS or C24, using the "floral dip" transformation protocol (Clough and Bent, 1998).

2.2.2 *MEA/FIS1*, *FIS2* and *FIE/FIS3* promoter::*GUS* expression pattern in hypomethylation condition

The transgenic plants carrying the *FIS* class gene::*GUS* fusions were crossed with a C24 transformed with anti- DNA *METHYTRANSFERASE*

I (Finnegan et al., 1997). These F1 plants were examined for *GUS* expression both maternally and paternally. These F1 plants should be hypomethylated, because all these F1 plants were able to rescue the *fis* maternal defects when used as pollen donors (See next chapter for more detail).

2.2.3 Microscopy and GUS Staining

After GUS staining (De Block and De Brower, 1992), ovules were directly cleared with Lactophenol and observed with differential interference contrast microscopy.

2.3 Results

2.3.1 Patterns of activity of the seed repressor genes

The *FIS2*, *FIE/FIS3,* and *MEA/FIS1*::*GUS* fusion proteins were each expressed in the female gametophyte before pollination and in the developing endosperm after pollination (Fig. 2.1, 2.2 & 2.3). *FIS2*::*GUS* activity (Fig. 2.1) was first evident in the unfused polar nuclei (Fig. 2.1 A) and subsequently in the nucleus of the central cell in the embryo sac (Fig. 2.1 B). After fertilization, *FIS2*::*GUS* activity was associated with the primary endosperm nucleus and subsequently with the dividing endosperm nuclei (Fig. 2.1 C-G). *FIS2*::*GUS* activity continued to be associated with the free endosperm nuclei until a cenocytic cyst formed in the chalazal region of the embryo sac (Fig. 2.1 H & I). *FIS2*::*GUS* activity in the free endosperm nuclei ceased before the time of endosperm cellularisation but continued in the nuclei of the chalazal cyst. *FIS2*::*GUS*

activity was occasionally observed in the synergids and the egg apparatus in a small proportion of the unfertilized embryo sacs (Fig. 2.1 J and K).

The activity pattern of *MEA/FIS1*::*GUS* was similar to that of *FIS2*::*GUS,* but the localization around the nuclei was more diffuse (Fig. 2.2 A-F). *MEA/FIS1*::*GUS* protein, before pollination, occurred in the fused polar cells (Fig. 2.2 A). After fertilization, *MEA/FIS1*::*GUS* activity was associated with dividing endosperm nuclei (Fig. 2.2 B-E), and the final pattern of activity was similar to that of *FIS2*::*GUS* plants, with GUS activity maintained in the chalazal cyst but ceasing in the other nuclei before endosperm cellularisation (Fig. 2.2 F). In a small number of embryo sacs GUS staining was observed in some nuclei at the micropylar end of the embryo sac at the time of endosperm cellularisation (data not shown). Vielle-Calzada et al. (1999) observed *MEA/FIS1* expression in both embryo and endosperm after pollination.

FIE/FIS3::*GUS* protein was diffuse throughout the embryo sac, and before anthesis was observed in the developing pollen microspores (Fig. 2.3 A, B, C and D); this activity was transient and was not evident in mature pollen (Fig. 2.3 E). The diffuse distribution of product of the *FIFIS3E*::*GUS* construct in the embryo sac persisted after fertilization and was observed in both the uncellularised/cellularised endosperm and embryo of heart stage (Fig. 2.3 F-J). GUS activity was observed in some sporophytic tissues: leaves, silique wall, and stamen filaments (data not shown).

Thus in accordance with their mutant phenotypes, *MEA/FIS1*, *FIS2,* and *FIE/FIS3* are expressed in the female gametophyte before pollination. However, in contrast to *MEA/FIS1* and *FIS2*, the *FIE/FIS3* product is

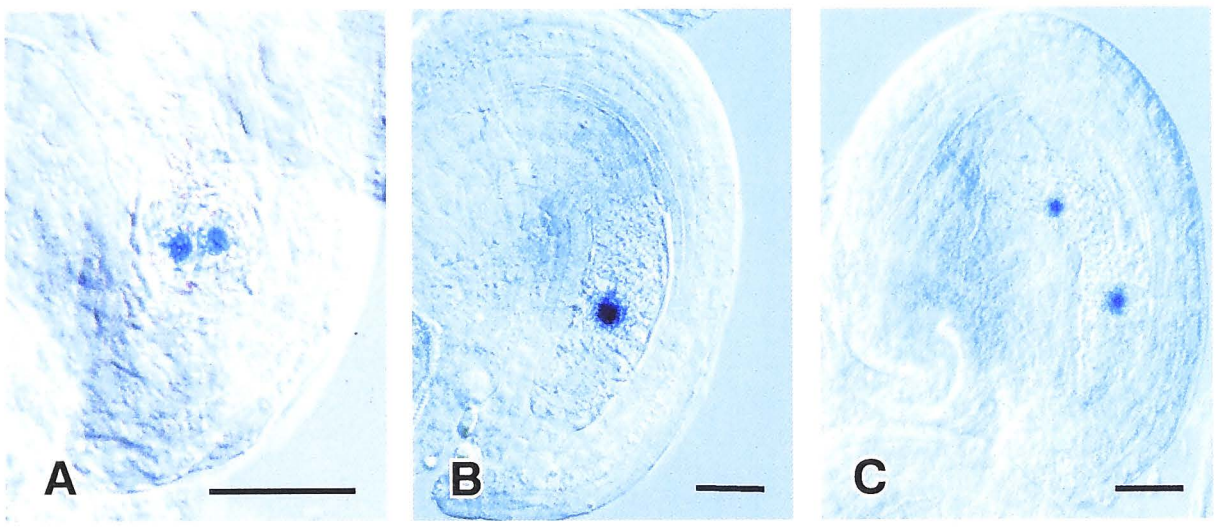


Fig. 2.1 *FIS2*::*GUS* activity. (A&B) Unpollinated ovules showing GUS activity in the central cell nucleus. (C-G) Free endosperm nuclei divide from two to 32. (H&I) Nuclei in cyst region retaining GUS activity. (J&K) Synergids and possible egg nuclei showing GUS activity. (Bar=0.05mm.)

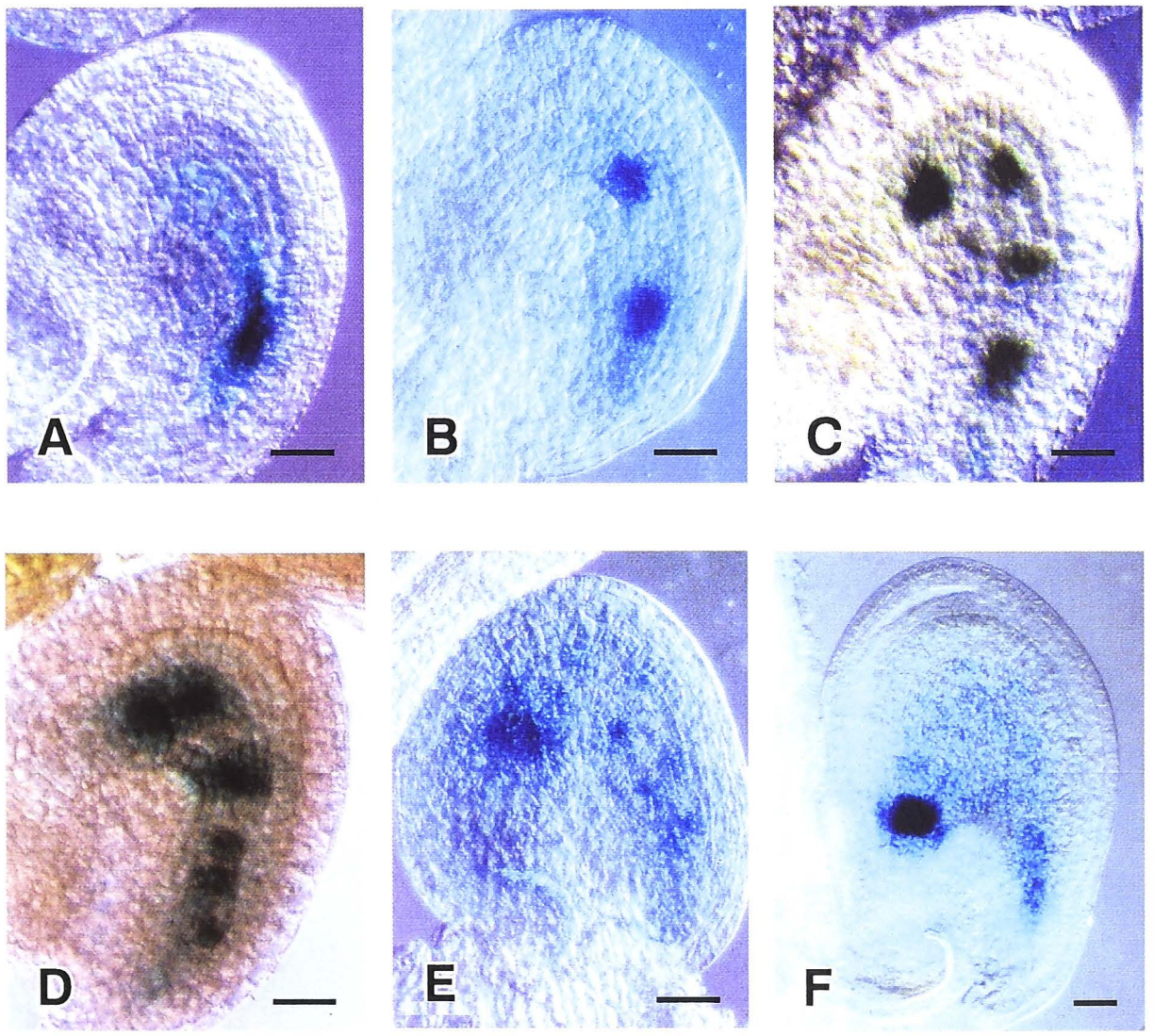


Fig. 2.2 *MEA/FIS1*::*GUS* activity. (A) Unpollinated ovule showing GUS activity in the central cell nucleus. (B-E) *MEA/FIS1*::*GUS* activity in dividing endosperm nuclei. (F) Nuclei in cyst region retaining GUS activity. (Bar=0.05mm.)

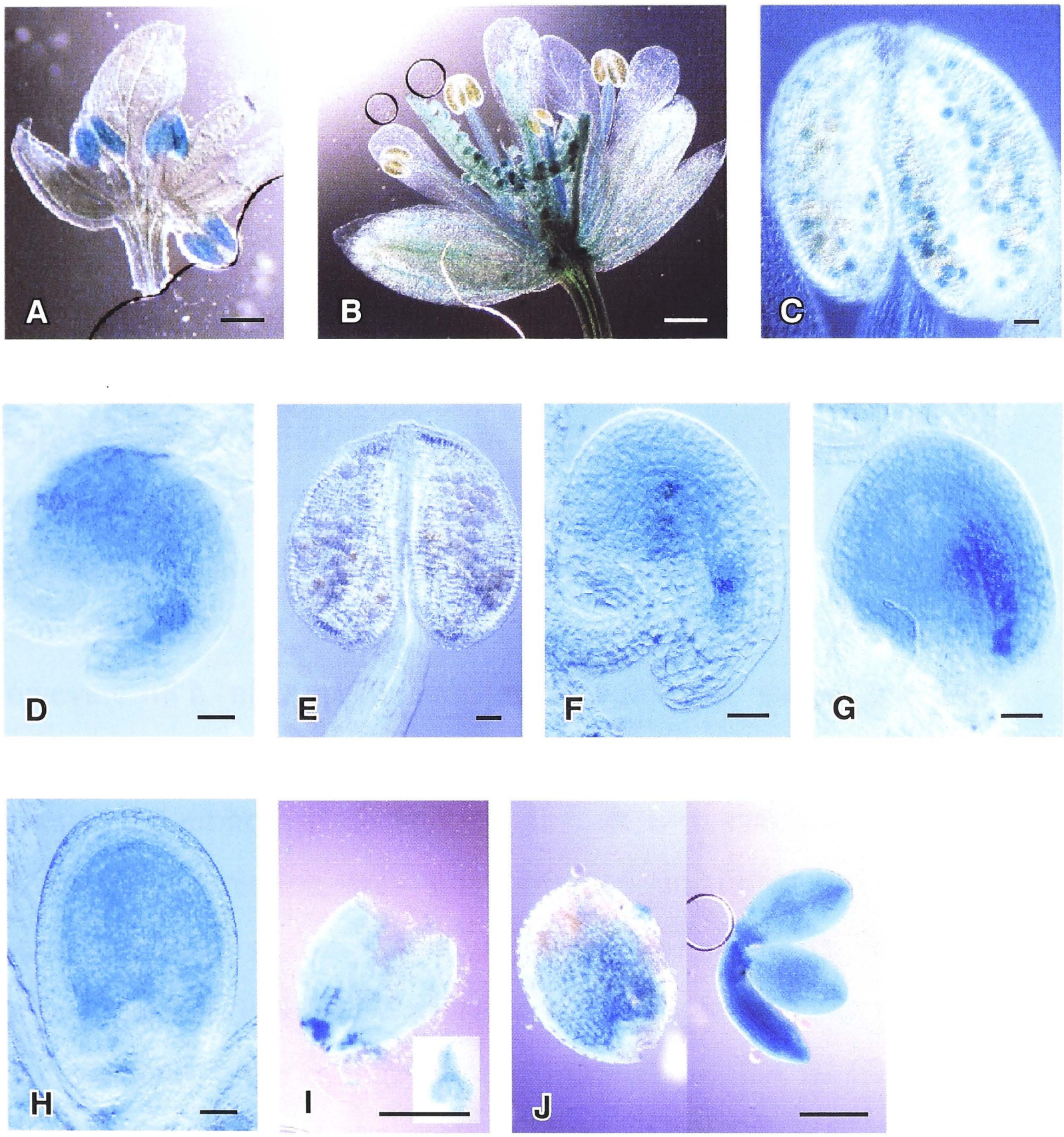


Fig. 2.3 *FIE/FIS3*::*GUS* activity. (A-C) Microspores showing transient GUS activity. (D) Unpollinated ovule showing GUS activity. The central cell region is dense. (E) Mature pollens showing no GUS activity. (F-H) Ovule 24h-48h after pollination, showing diffuse GUS activity. (I&J) Three-day-old and seven-day-old seeds dissected to stain embryo and rest of the seed separately, showing the GUS activity in embryo and inside the seed coat. (Bar=0.017mm in A, B, C, E, I & J.) (Bar=0.05mm in D, E, F, G & H.)

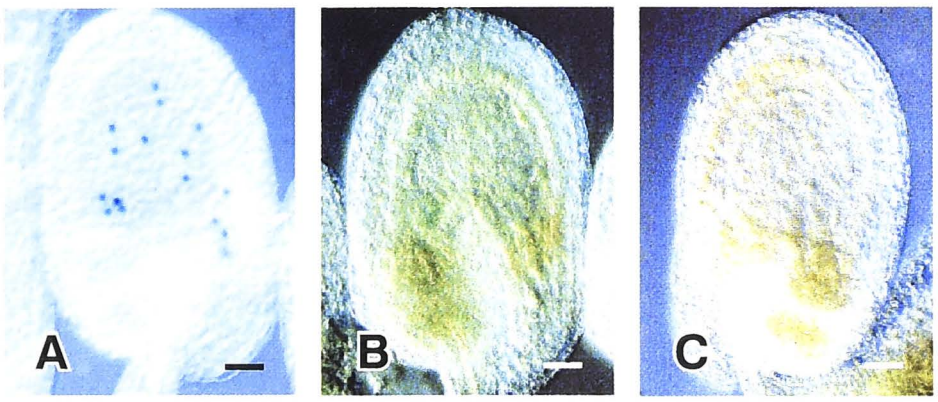
found not only in the endosperm but also in the embryo and other sporophytic tissues, indicating that *FIE* might have functions other than those in endosperm development. Our inability to make a *fie/fie* homozygote also indicates a critical requirement for this gene in the zygote.

2.3.2 Activity of the *FIS* genes depends on the parent of origin

The effect of parental origin of the *FIS* genes was also examined using *FIS::GUS* fusions. In contrast to the endosperm-specific activity of the maternally derived *FIS2*::*GUS* transgene (Fig. 2.4 I A), no product was observed when *FIS2*::*GUS* was introduced by pollen (Fig. 2.4 I B & C). Similarly, there was no GUS protein during the early stages of endosperm formation when *MEA/FIS1*::*GUS* was introduced by pollen (Fig. 2.4 II B). However, 48 h after pollination (early globular stage), paternal *MEA/FIS1*::*GUS* expression was observed in the chalazal cyst and sometimes in the embryo itself (Fig.2.4 II C & D). Paternally derived *FIE/FIS3*::*GUS* expression was not observed in either embryo or endosperm until 3 days after pollination (Fig. 2.4 III A & B). At 4 days after pollination, *FIE/FIS3*::*GUS* expression was observed in early heart-stage embryos and in the cellularised endosperm (Fig. 2.4 III C and D). Because the maternal and paternal expression of *FIE/FIS3*::*GUS* in embryo occurred around the same stage, it is likely that *FIE/FIS3* was not imprinted in the embryo but was in the endosperm. There was no expression of paternally derived *FIS2*::*GUS* at any developmental stage in either ovules or seeds.

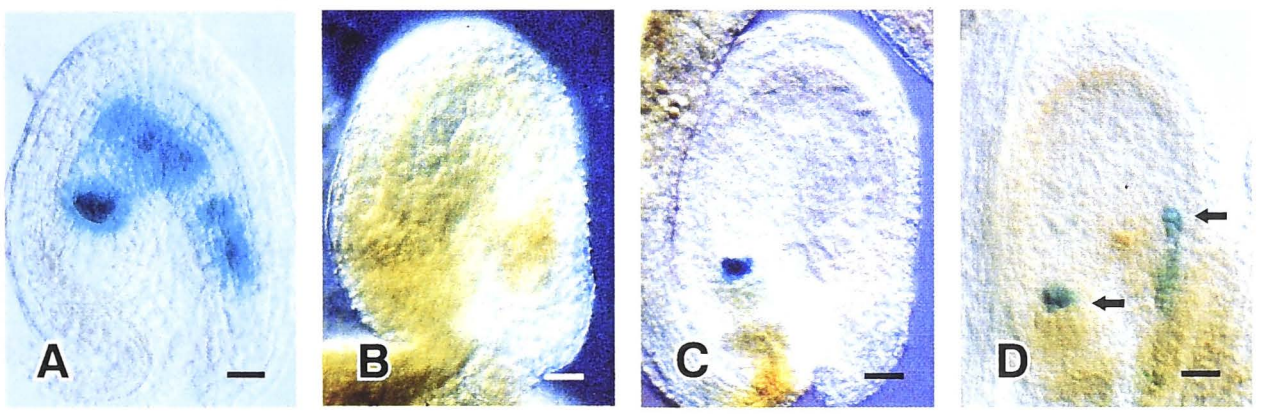
Our data show that *MEA/FIS1* is imprinted during early endosperm development but that later (48 hour) in endosperm development the

I *FIS2* Imprinting



Maternal *FIS2*::*Gus* Paternal *FIS2*::*Gus*

II *MEA/FIS1* Imprinting



Maternal *MEA*::*Gus* Paternal *MEA*::*Gus*

III *FIE/FIS3* Imprinting

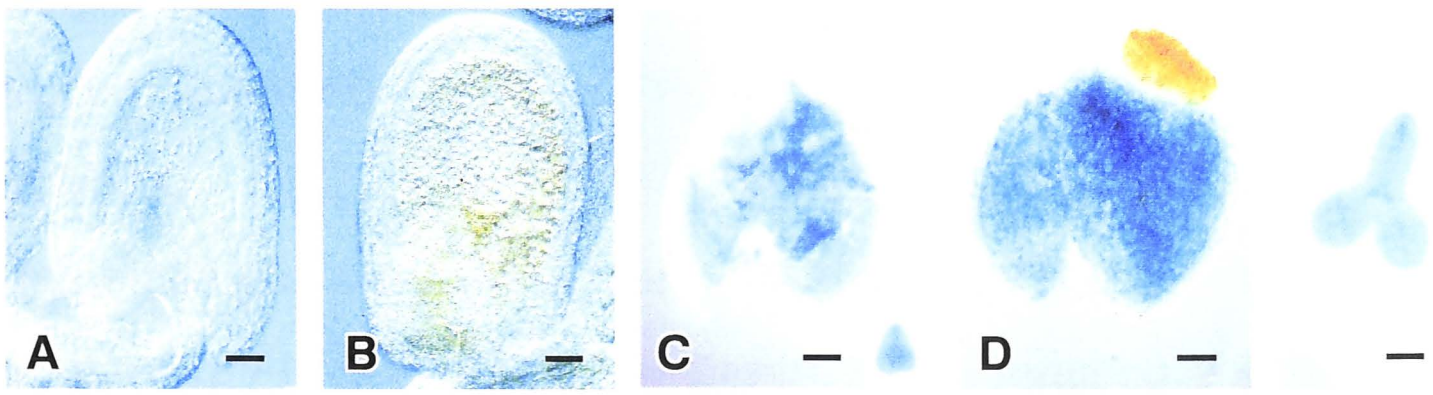


Fig. 2.4 *FIS2, MEA/FIS1,* and *FIE/FIS3* imprinting. (I A) 24-hour-old ovule with maternally derived *FIS2*::*GUS,* showing GUS activity. (I B&C) 24-hour-old and 48-hour-old L*er* ovules pollinated with a *FIS2*::*GUS* plant, showing no GUS activity. (II A) 24-hour-old ovule with maternally derived *MEA/FIS1*::*GUS,* showing GUS activity. (II B) 24-hour-old L*er* ovule pollinated with a *MEA/FIS1*::*GUS* plant, showing no GUS activity. (II C&D) 48-hour-old ovule showing GUS activity in cyst and possibly in embryo after pollination with *MEA/FIS1*::*GUS.* (III A&B) No GUS activity seen in a L*er* ovule pollinated with *FIE/FIS3*::*GUS* 24h and 48h after pollination. (III C&D) Endosperm and heart and torpedo stage embryos showing GUS activity in an ovule pollinated by a *FIE/FIS3*::*GUS* plant four days and seven days after pollination. (Bars = 0.05mm.)

imprinting breaks down, with *MEA/FIS1*::*GUS* being expressed in the chalazal cyst. I also observed embryo expression of paternally derived *MEA/FIS1*::*GUS* genes at low frequency at 48 h after pollination. Earlier, Kinoshita et al. (1999) showed imprinting only in the endosperm expression, and Vielle-Calzada et al. (1999) showed imprinting in both embryo and endosperm, using reverse transcriptase-PCR analyses. My method, using the sensitive GUS reporter gene, indicates that the imprinting of *MEA/FIS1* shows both a spatial and a temporal pattern. I have further shown that all three *FIS* genes show maternal expression in the developing seed, and each has a unique time of cessation of activity as seed development proceeds.

2.3.3 Maternal expression and paternal imprinting of *FIS2*, *MEA/FIS1*, and *FIE/FIS3*::*GUS* were not altered in hypomethylation condition.

Since DNA methylation is involved in the imprinting control of genes in the animal kingdom (Sanford et al., 1987), I checked the expression pattern of *MEA/FIS1*, *FIS2*, and *FIE/FIS3* promoters::*GUS* protein fusions under hypomethylation condition by crossing the GUS positive plants to C24 anti-*MET1*, which has a significantly reduced DNA methylation level (Finnegan et al., 1997). The expression pattern of those F1 plants was investigated. I expected that the paternal silencing of *FIS1/MEA*, *FIS2* and *FIE/FIS3*::*GUS* gene would be lifted if DNA methylation controls paternal imprinting, and the maternal expression pattern would not change.

The *FIS2*::*GUS* fusion proteins under hypomethylation condition were all expressed in the female gametophyte before pollination and in the

developing endosperm after pollination in the same way as in plants with normal levels of methylation. *FIS2*::*GUS* activity under hypomethylated plants was first evident in the unfused polar nuclei and subsequently in the nucleus of the central cell in the unfertilised embryo sac (Fig. 2.5 A). After fertilization, *FIS2*::*GUS* activity was seen within the primary endosperm nucleus and subsequently with the dividing endosperm nuclei (Fig. 2.5 B-E). *FIS2*::*GUS* activity continued to be associated with the free endosperm nuclei until a cenocytic cyst formed in the chalazal region of the embryo sac (Fig. 2.5 F) 48 hour post fertilisation. *FIS2*::*GUS* activity in the free endosperm nuclei ceased before the time of endosperm cellularisation but continued in the nuclei of the chalazal cyst. *FIS2*::*GUS* activity was occasionally observed in the synergids in a small proportion of the unfertilized embryo sacs (Fig. 2.5 A). In contrast to the endosperm-specific activity of the maternally derived *FIS2*::*GUS* transgene under either normal methylation or hypomethylation (Fig. 2.1 C-G & 2.5 B-E), no GUS product was observed from a wild type plant when the *FIS2*::*GUS* transgene was introduced via pollen of the hypomethylated *FIS2*::*GUS* plants (Fig. 2.5 G & H) in 24 and 48 hour old seeds. The *FIS2*::*GUS* expression pattern was not altered under hypomethylation condition.

The activity pattern of *MEA/FIS1*::*GUS* in hypomethylated plants was similar to that of *FIS2*::*GUS*, but the localization around the nuclei was still diffuse (Fig. 2.6). *MEA/FIS1*::*GUS* activity, before pollination, occurred in the fused polar cells (Fig. 2.6 A). After fertilization, *ME/FIS1*::*GUS* activity was associated with dividing endosperm nuclei (Fig. 2.6 B-D), and the final pattern of activity was similar to that of *FIS2*::*GUS* plants, with GUS activity maintained in the chalazal cyst but ceasing in the other nuclei before endosperm cellularisation (Fig. 2.6 E).

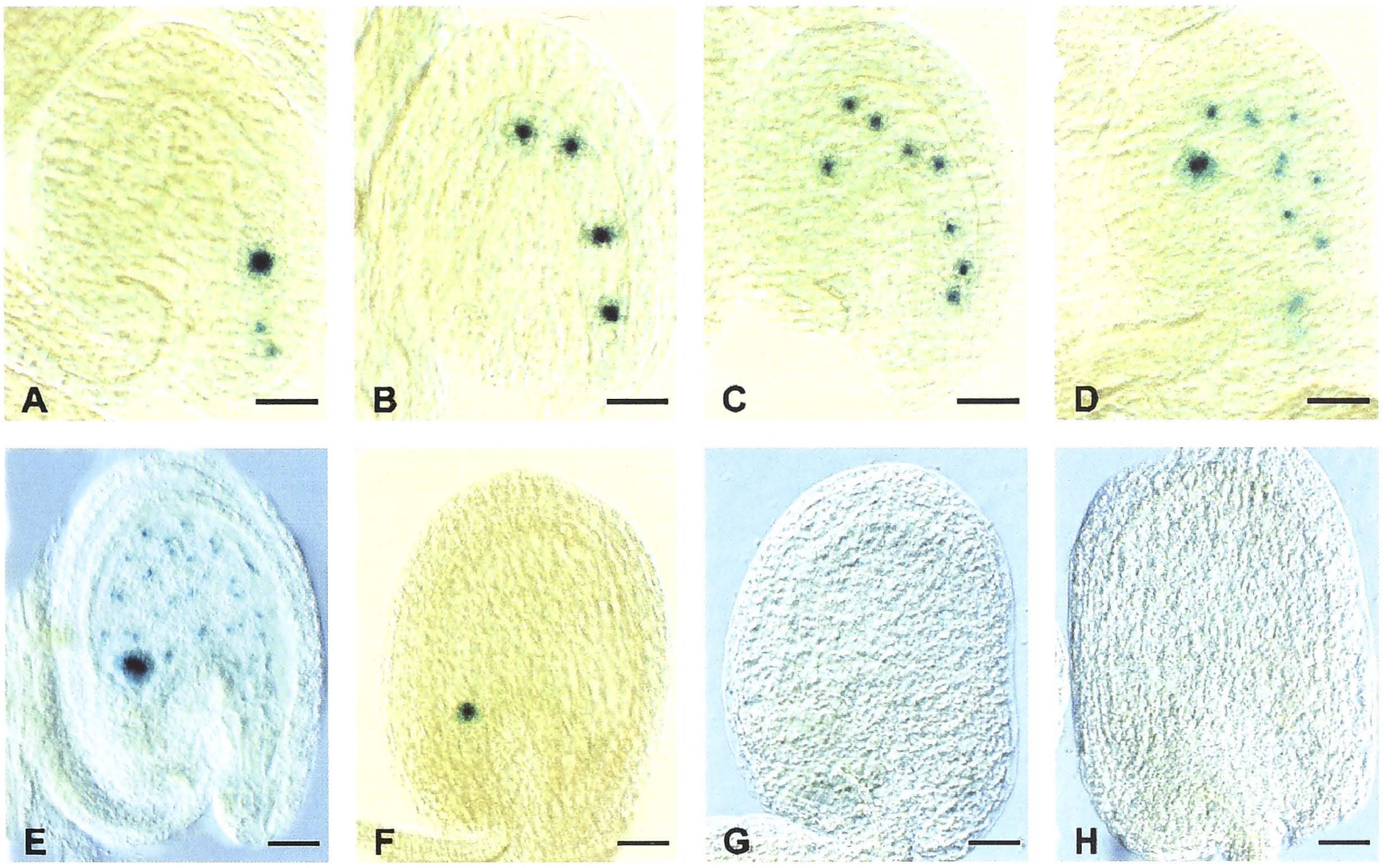


Fig. 2.5 *FIS2::GUS* activity in hypomethylation. (A) Unpollinated ovules showing GUS activity in the central cell nucleus and sometimes in synergids. (B-E) Free endosperm nuclei divide from four to 32. (F) Nuclei in cyst region retaining GUS activity. (G&H) 24-hour-old and 48-hour-old L*er* ovules pollinated with a hypomethylated *FIS2::GUS* plant, showing no GUS activity. (bar=0.05mm.)

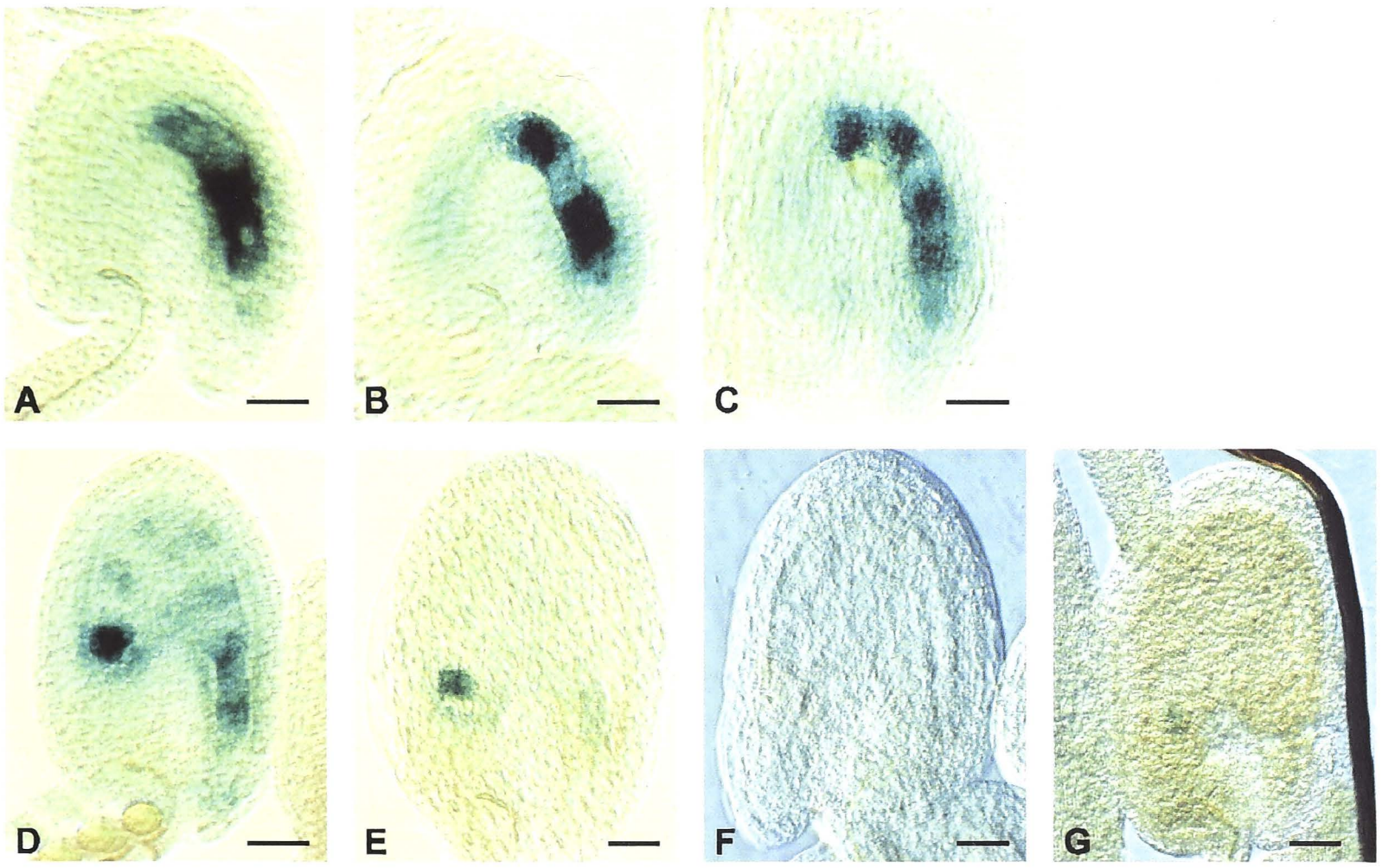


Fig. 2.6 *MEA/FIS1::GUS* activity in hypomethylation. (A) Unpollinated ovule showing GUS activity in the central cell nucleus. (B-D) *MEA*::*GUS* activity in dividing endosperm nuclei. (E) Nuclei in cyst region retaining GUS activity. (F) 24-hour-old L*er* ovule pollinated with a hypomethylated *MEA/FIS1*::*GUS* plant, showing no GUS activity. (G) 48-hour-old L*er* ovule showing weak GUS activity in cyst pollinated with a hypomethylated *MEA/FIS1*::*GUS* plant (bar=0.05mm.)

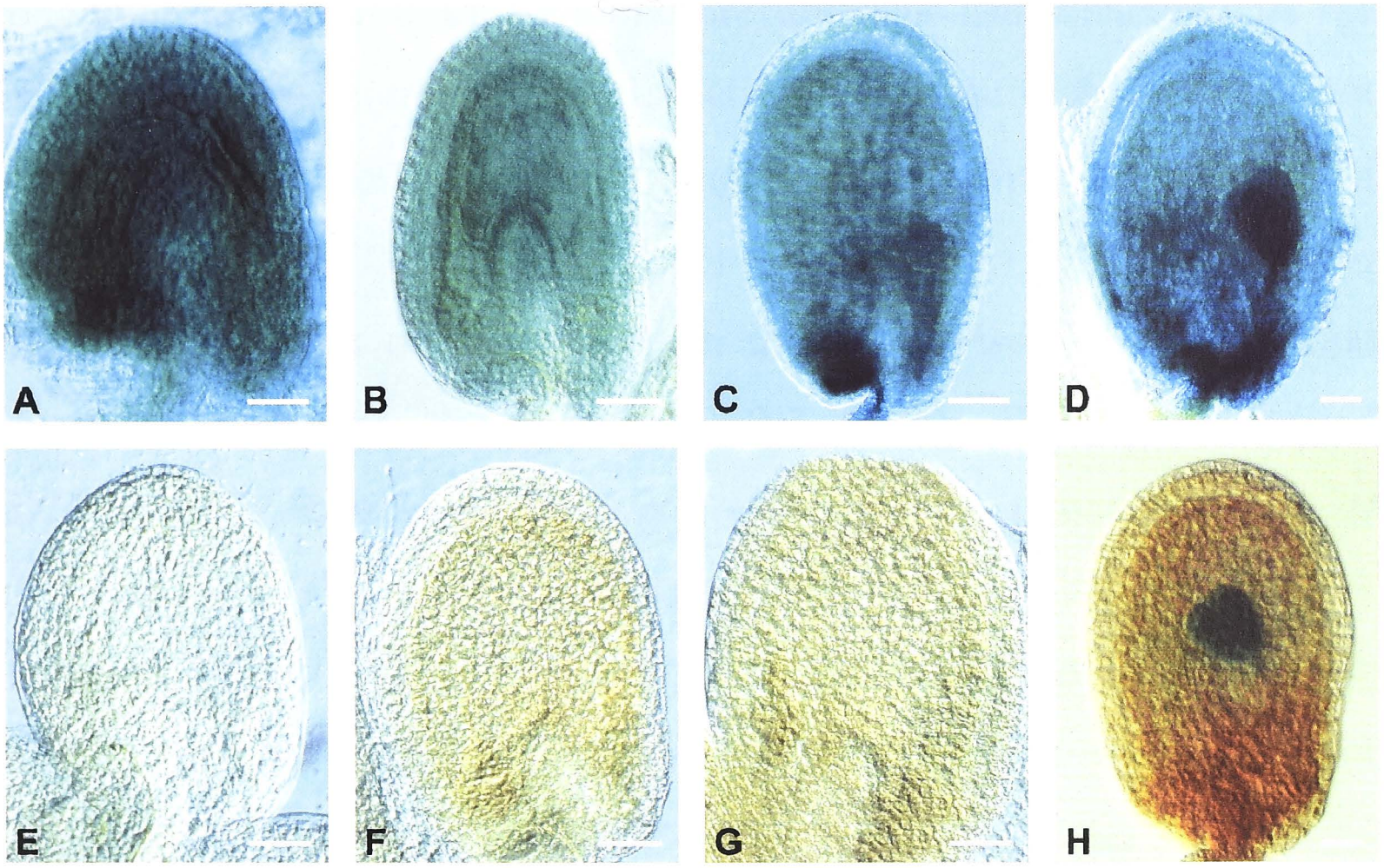


Fig. 2.7 *FIE/FIS3::GUS* activity in hypomethylation. (A) Unpollinated ovule showing GUS activity. (B-C) Ovules 24h-48h after pollination, showing diffuse GUS activity. (D) four-day-old seeds, showing the GUS activity in embryo and endosperm. (E-G) No GUS activity seen in a L*er* ovule pollinated with hypomethylated a *FIE/FIS3*::*GUS* plant, 24h, 48h and 72h after pollination. (H) Heart stage embryo showing GUS activity in an ovule pollinated by a hypomethylated *FIE/FIS3*::*GUS* plant 4 days after pollination. (bar=0.05mm.)

Similarly, there was no *MEA/FIS1*::*GUS* activity during the early stages of endosperm formation in a witd type plant when *MEA/FIS1*::*GUS* was introduced via pollen of the hypomethylated *MEA/FIS1*::*GUS* plants (Fig. 2.6 F). 48 h after pollination (early globular stage), paternal *MEA/FIS1*::*GUS* expression was observed in the chalazal cyst as in the wild type plant (Fig. 2.6 G).

In the hypomethylated plants *FIE/FIS3*::*GUS* protein was diffuse throughout the embryo sac, and before anthesis was observed in the developing pollen microspores; this activity was transient and was not evident in mature pollen (Fig. not shown). The diffuse distribution of product of the *FIE/FIS3*::*GUS* construct in the embryo sac persisted after fertilization and was observed in both the cellularised endosperm and embryo at early heart stage (Fig. 2.7 A-D). Paternally derived *FIE/FIS3*::*GUS* expression was not observed in either embryo or endosperm until 3 days after pollination (Fig. 2.7 E-G). At 4 days after pollination, *FIE/FIS3*::*GUS* expression was observed in early heart-stage embryos (Fig. 2.7 H). The maternal and paternal expression of *FIE/FIS3*::*GUS* in hypomehtylated plants is the same as that in the normal methylated plants except that I did not observe the imprinting breakdown in endosperm, which occurred in the wild type plants pollinated with a *FIE/FIS3*::*GUS* plant with normal levels of methylation.

2.4 Discussion

2.4.1 Expression of the *FIS* genes is consistent with their function.

The genes *MEA*, *FIS2,* and *FIE* repress seed development until the double fertilization event that follows pollination provides the signals for embryo

and endosperm development. The experiments, in which I coupled the promoters of the three genes to the *GUS* reporter gene, have shown that after fertilization, *MEA/FIS1*, *FIS2,* and *FIE/FIS3* activities can be detected in the endosperm tissue and that *FIE/FIS3* activity also occurs in other sporophytic tissues, indicating that *FIE/FIS3* has multiple functions rather than just in endosperm for *Arabidopsis* development. Kinoshita et al. (2001) reported that FIE-mediated polycomb complexes are an essential component of a floral repression mechanism established early during plant development. Both the maternally and paternally derived GUS expression was seen in the heart staged embryo, indicating that the *FIE/FIS3* has a sporophytic function in embryo development (Luo et al., 2000). This conclusion is supported by the evidence that no *fie* homozygous plant has been isolated yet (Chaudhury et al., 1997).

Before pollination the *FIS* genes are expressed in the female gametophyte: *MEA/FIS1* and *FIS2* products are found in the two polar cells, in the central cell, and in all of the dividing cells of the endosperm. The *FIE/FIS3*::*GUS* fusion product also occurs in the central cell before pollination as well as in the developing endosperm. The expression of these genes in the polar nuclei and in the central cell could be necessary for the repression of endosperm development and of other processes in seed development; it is likely, given the sequence affinities of these genes, that repression is mediated by a polycomb complex involving MEA/FIS1 and FIE/FIS3 directly, with the FIS2 protein functioning as a related member. The physical interaction between MEA/FIS1 and FIE/FIS3 proteins has been demonstrated (Luo et al., 2000; Spillane et al., 2000).

The activity patterns of all three genes have demonstrated that there are differentiated regions of the endosperm. *MEA/FIS1* and *FIS2* activity continue in the cenocytic cyst in the chalazal end of the endosperm after the activity of these genes ceases in the central and micropylar regions of the embryo sac. The cenocytic condition of the chalazal cyst continues beyond the initiation of cellularisation of the remainder of the endosperm, which may also be a consequence of the presence or absence of the *MEA/FIS1* and *FIS2* gene products.

2.4.2 Imprinting of *FIS* genes

It has been shown that each of these genes shows a parent-of-origin effect; paternally derived wild-type genes are not able to rescue the maternally derived lesion in the corresponding gene (Chaudhury et al., 1997; Ohad et al., 1997). One interpretation of this observation is that the genes are imprinted and are not expressed when derived paternally (Kinoshita et al., 1999; Vielle-Calzada et al., 1999). Our experiments have provided strong evidence that all three *FIS* genes are regulated in a similar way that the gene copies from pollen are not expressed in the early development of the endosperm. Because the wild type pollen carries the silenced (or imprinted) *FIS* genes copies, the ovules carrying the *fis* mutations will give aborted seeds either pollinated with wild type pollen or mutant pollen. Moreover, *FIE*::*GUS* is expressed in heart-staged embryo maternally and paternally, indicating that *FIE* exerts a sporophytic function in embryo development but a gametophytic one in endosperm development.

2.4.3 Imprinting is not controlled by DNA-methylation

Since DNA methylation is involved in the imprinting control of genes in animal kingdom (Sanford et al., 1987), I checked the expression pattern of *MEA/FIS1*, *FIS2*, and *FIE/FIS3* promoters::*GUS* protein fusion under hypomethylation condition by crossing the GUS positive plants to C24 anti-*MET1*, which has a significantly reduced DNA methylation levels (Finnegan et al., 1997). I expected that the paternal silencing of *MEA/FIS1*, *FIS2* and *FIE/FIS3*::*GUS* gene would be lifted in the F1 generation if DNA methylation controls paternal imprinting, and the maternal expression pattern would not change.

However I have shown that the imprinting of *FIS* genes is not related to the level of genome methylation in the female or the male, and therefore it is not likely that the regulation of these genes is dependent on DNA methyltransferase activity. There must be mechanisms other than DNA-methylation controlling imprinting of *FIS* genes. DEMETER, a DNA glycosylase domain protein, is required for *MEA/FIS1* imprinting and seed viability in *Arabidopsis* (Choi. et al; 2002). It activates the maternal *MEA/FIS1* copy during early seed development. Whether DEMETER also controls FIS2 or FIS3/FIE imprinting is not known.

CHAPTER 3

The effect of DNA methylation on seed development

3.1 Introduction

DNA methylation plays an essential role in regulating plant development. Genome wide demethylation has a pleiotropic effect on plant morphology, including homeotic transformations in floral organs and altered flowering time (reviewed by Finnegan et al., 2000). DNA demethylation imposed by anti-*MET1* might create epigenetic alleles, of which some result in phenotypic change. The ectopic hypermethylation of the *SUPERMAN* gene has been reported in the same C24 anti-*MET1* line used for my experiments (Kishimoto et al., 2001). A second floral development gene, *AGAMOUS*, also became hypermethylated and silenced in the same line (Jacobsen et al., 2000). These results suggest that hypermethylation of specific genes in backgrounds that show low genome-wide levels of DNA methylation may explain some of the developmental defects seen in *Arabidopsis* methylation deficient mutants. This resembles a phenomenon seen in cancer cells, which can simultaneously show genome-wide hypomethylation and hypermethylation of specific genes.

The loss of function of *FIS* class genes in *Arabidopsis* causes two different phenotypes: autonomous endosperm development in the absence of fertilization, and following fertilization, an arrested embryo with over-proliferation of the endosperm which is not cellularised (Chaudhury et al., 1997; Ohad et al., 1997). The genetic observations in which reciprocal crosses involving wild-type and mutant alleles of the three *FIS* genes did

not give identical results indicated a parent-of-origin effect of the *FIS* mutation; the egg carrying a *fis* mutation gives an aborted embryo and the egg carrying the *FIS* wild type allele gives a normal embryo irrespective of the sperm genotype (Chaudhury et al., 1997; Ohad et al., 1997; Grossliknaus et al., 1998). As shown in the last chapter, the expression of *MEA/FIS1*, *FIS2* and *FIE/FIS3* in the developing endosperm is imprinted; the maternal copies of *FIS* class genes are active and the paternal copies are silent in early developing endosperm. The maternal expression and paternal imprinting of *FIS2*, *MEA/FIS1*, and *FIE/FIS3*::*GUS* were not altered in plants with low levels of DNA methylation.

Hypomethylated pollen can rescue the post-fertilization embryo arrest of *mea/fis1*; the *mea/fis1* phenotype can be rescued by the inclusion of a *ddm1* mutant allele in the paternal plant (Vielle-Calzada et al., 1999). *ddm1* is associated with reduced methylation levels and chromatin restructuring (Jeddel et al., 1999). Vielle-Calzada et al. (1999) suggest that the rescue of the arrested-seed phenotype is caused by the reactivation of the paternally derived wild-type *MEA/FIS1* allele in the developing seed, the reactivation being dependent on the reduced methylation level or on chromatin restructuring caused by the *ddm1* allele.

In this chapter, I test whether the maternal defect of the *fis* class mutants can be rescued by pollen from low methylation plants and if DNA hypomethylation is involved in parent-of-origin effects on seed development.

3.2 Material and methods

3.2.1 Plant materials

Mea/mea (*fis1/fis1*), *fis2-3/fis2-3*, *FIE2/fie2* (*FIS3-1/fis3-1*) and C24 anti-*MET1* were used in this experiment (Chaudhury et al., 1997). C24 anti-*MET1* was a gift from Dr. Jean Finnegan et al. (1997). *ddm1* mutant was obtained from *Arabidopsis* Stock Centre. Growth condition followed Luo et al. (2000).

3.2.2 Primers and sequencing

The primers for *mea/fis1* gene are 5'-ccagcccgctgcagag-3' and 5'-ctttcagccatcagctgg-3' (Luo et al., 1999). The PCR products were cut with *Mse* I for mutation detection, because the fragment from mutant *mea/fis1* allele has another *Mse* I site caused by mutation. The primers for *fis2* are 5'-aacatcatggtgagttcaac-3' and 5'-tttaaatttgagttttatgtaag-3'. The primers for *fie* are 5'-cattgcaatcctatgctgat-3' and 5'-cagtaatcacaagttgaggttt-3'. The PCR products were to be sequenced for detecting *fis2* or *fie/fis3* mutations. The PCR condition was 95℃ for 5 minutes for 1 cycle, then 35 cycles of 95℃ for 15 seconds, 50℃ for 15 second and 72℃ for 35 second. The PCR products for *fie/fis3* and *fis2* are used for sequencing to detect the mutations.

The PCR products were sequenced by an Applied Biosystems Model 370A DNA Sequencer with fluorescent dye-labelled dideoxy terminators.

3.2.3 Genetic Mapping

For genetic mapping, the information about the sslps distinguishing C24 and L*er* can be obtained from the http://www.arabidopsis.org. Another marker, F3996FR, was designed based on the sequence difference between Col. and L*er* on BAC T32N15 (Cereon Indel data base, http://www.arabidopsis.org). There is a deletion at position 39963bp of BAC T32N15 in C24 compared with L*er* sequence. A pair of PCR primers, 5'-cttttgtgcacgacggaatct-3' and 5'-agctagggtttggcaacatt-3', was generated around this deletion. The PCR products have 15 bp difference in length and can be detected on a 4% Agarose gel.

3.2.4 Microscopy

The pictures were taken under a dissection microscope (Wild Heerbrugg, Leitz). Fertilized and unfertilized siliques were dissected under a dissecting microscope. For optical microscopy, tissue was prepared according to Craig and Miller (1984). For scanning electron microscopy, dissected ovaries were attached with colloidal graphite to a copper stub, frozen under vacuum, and examined according to Craig and Beaton (1996).

3.3 Results

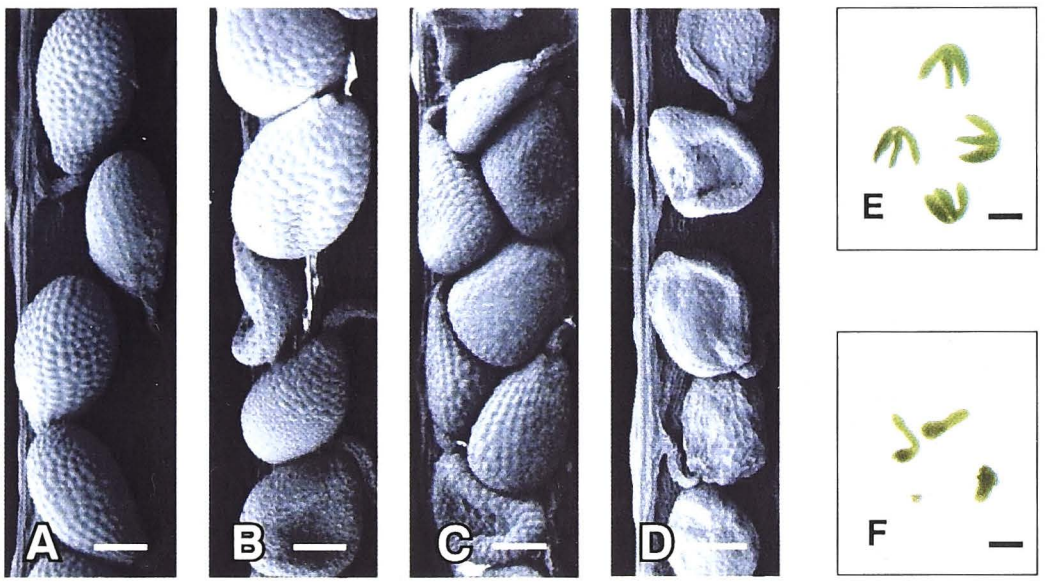
3.3.1 Arrested seed development in the *fis* mutants is rescued by pollen from hypomethylated plants.

When the homozygous *mea/fis1* and *fis2* mutants are pollinated with wild-type pollen, double fertilization occurs and both embryo and endosperm development are initiated. The developing endosperm fails to cellularise, and the syncytial nuclei continue to divide, expanding the

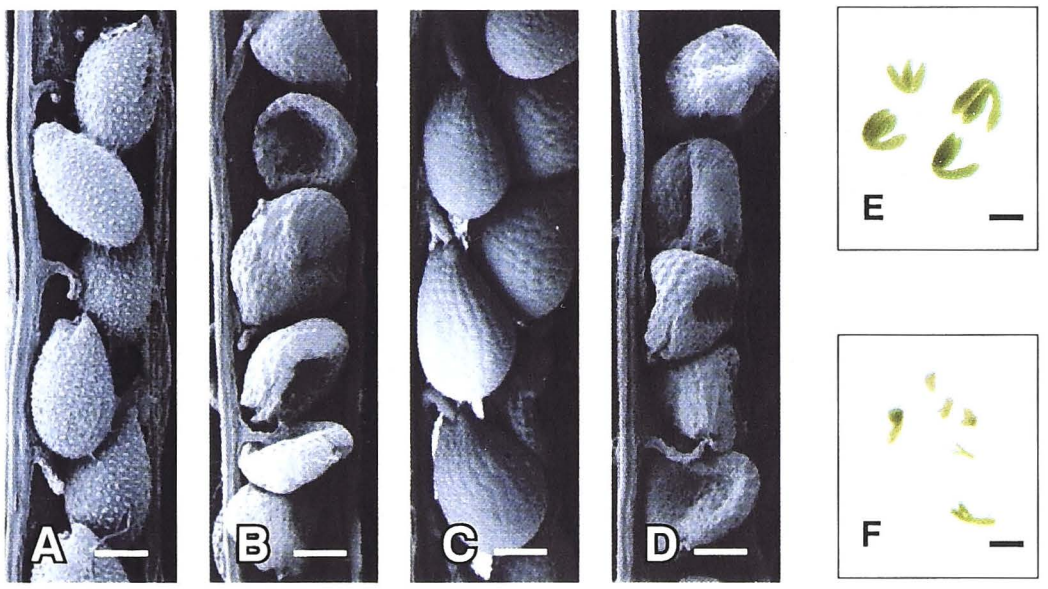
endosperm mass. The embryo develops normally but arrests at the heart/torpedo stage. Viable seeds are not formed (Chaudhury et al., 1997). A similar result was reported (Ohad et al., 1997) for the *fie/fis3* ovules in a *FIE/fie* heterozygote.

The aborted seeds of *mea/fis1* were rescued when pollinated by a line carrying the *ddm1* mutation (Vielle-Calzada et al., 1999), a recessive mutation in the *DECREASE IN DNA METHYLATION1* (*DDM1*) gene, *ddm1-2*, encoding a chromatin-remodelling SWI2/SNF2-like protein (Jeddeloh et al., 1999). To test the role of level of DNA methylation in the rescue of *mea/fis1*, *fis2,* and *fie/fis3* embryos, I pollinated homozygous *mea/fis1* and *fis2* plants with pollen from wild-type C24 plants and from C24 plants homozygous for the anti-*MET1* construct, in which DNA methylation levels are approximately 15% of the wild-type level (Finnegan et al., 1997). When *mea/fis1* homozygotes were pollinated with the anti-*MET1* pollen, 98.8% of the seeds were viable (Fig. 3.1 I A), and endosperm and embryo development were normal (Fig. 3.2 A), compared with 24% viable seed when the same *mea/fis1* plants were pollinated with wild-type C24 pollen (Fig.3.1 I B). When *mea/fis1* homozygotes were pollinated with the *ddm1* pollen, 71.1% of the seeds were viable (Fig. 3.1 I C), compared with 0.9% viable seed when *mea/fis1* plants were pollinated with wild-type Columbia pollen (Fig. 3.1 I D). When homozygous *fis2* plants were pollinated with C24 anti-*MET1* pollen, 99% of seeds were viable (Fig. 3.1 II A) whereas wild-type C24 pollen rescued only 1.9% of the affected seed (Fig. 3.1 II B). The rescued *fis2* seed contained normal endosperm and embryo (Fig.3.2 C). When homozygous *fis2* plants were pollinated with *ddm1* pollen, 70.5% of seeds were viable (Fig. 3.1 II C), whereas wild-type Columbia pollen rescued only 0.7% of the affected seed (Fig. 3.1 II D). I was unable to produce a *fie/fie*

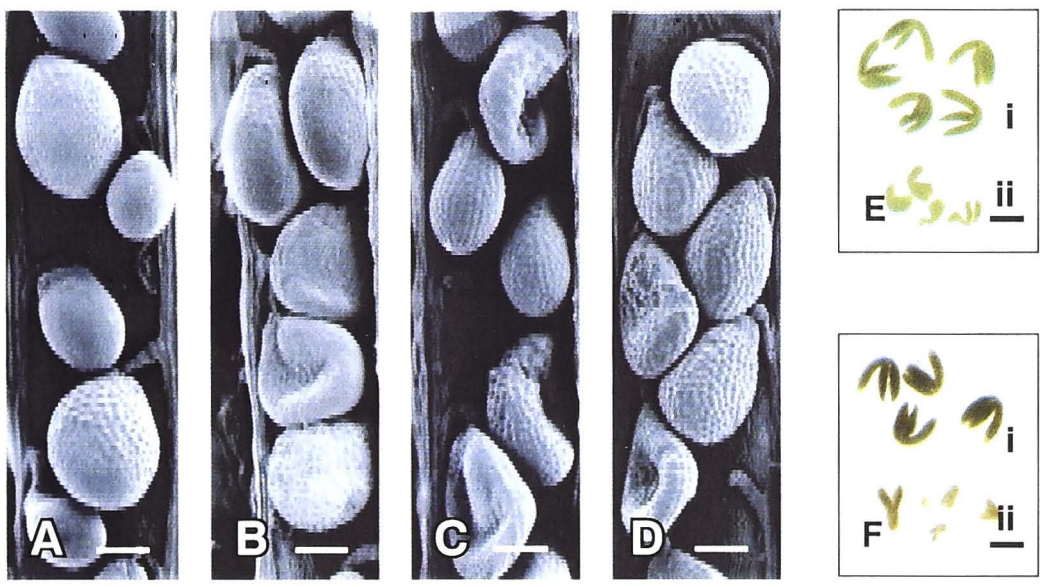
I *mea/fis1*



II *fis* 2



III *fie/fis3*



A: mutants x C24 anti-*MET1*, B: mutants x C24, C: mutants x *ddm1*, D: mutants x Col.

Fig. 3.1 *mea/fis1*, *fis2*, and *fie/fis3* seed rescued by C24 anti-*MET1* and *ddm1* pollen. (I A&E) *mea/mea* seeds and embryos pollinated by C24 anti-*MET1*. (I B&F) *mea/mea* pollinated with C24 pollen. Some seeds collapsed. Dissected embryos show arrest. (I C&D) *mea/mea* seeds pollinated by *ddm1* and Col pollen respectively. About 70% seeds were rescued. (II A&E) *fis2/fis2* seeds and embryos rescued with C24 anti-*MET1* pollen (II B&F) *fis2/fis2* pollinated with C24 pollen. Embryos are arrested. (II C&D) *fis2/fis2* seeds pollinated by *ddm1* and Col pollen respectively. About 70% seeds were rescued. (III A&E) *FIE/fie* pollinated with C24 anti-*MET1* pollen, showing two types of seeds: fully developed big seeds with big embryos (E i) and fully developed small seeds with small embryos (E ii). (III B&F) *FIE/fie* pollinated with C24 pollen. Half of the seeds are fully developed (F i) and others are aborted (F ii). (III C&D) *FIE/fie* seeds pollinated by *ddm1* and Col pollen respectively. About 50% seeds were small, other 50% aborted. (Bar=0.05mm.)

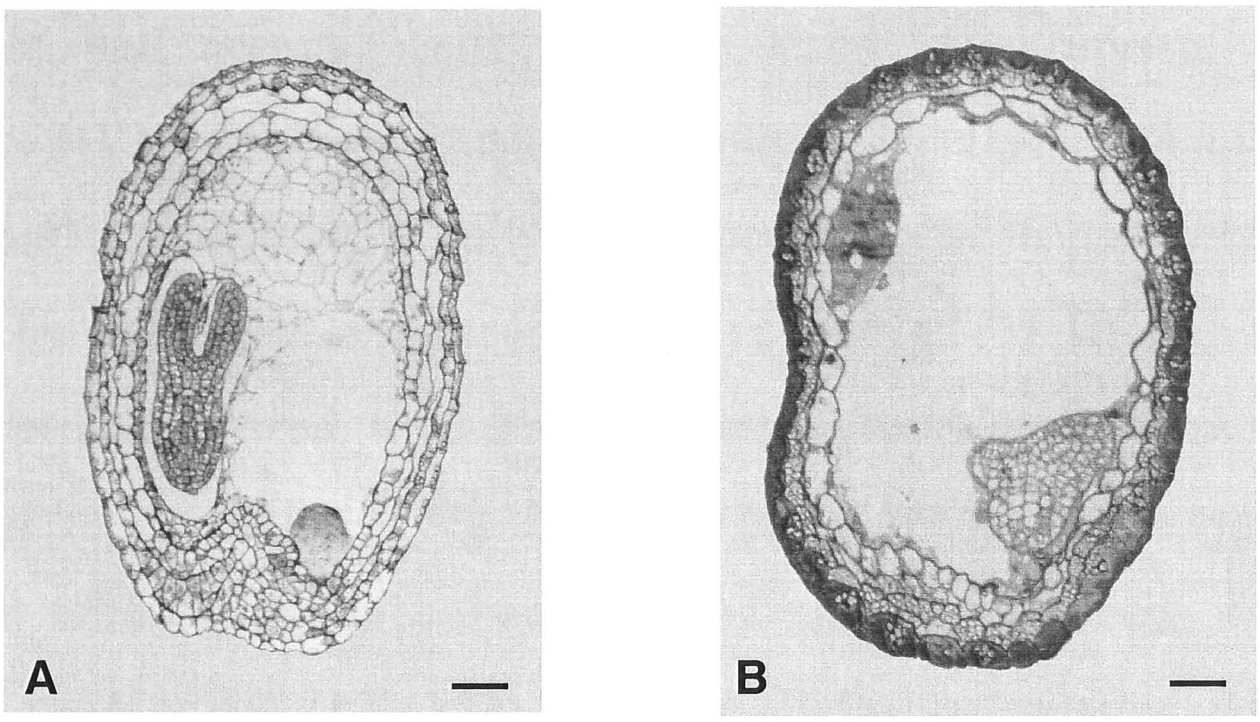


Fig. 3.2 sections of *fis* rescued seeds and non-rescued seeds. (A) *mea/mea* five-day-old seed rescued by C24 anti-*MET1* with cellularised endosperm and torpedo embryo. (B) *mea/mea* selfed five-day-old seed with arresting embryo and uncellularised endosperm. (C) *fis2/fis2* five-day-old seed rescued by C24 anti-*MET1* with cellularised endosperm and torpedo embryo. (D) *fis2/fis2* selfed five-day-old seed with arresting embryo and uncellularised endosperm. (E) *FIE/fie* five-day-old seed rescued by C24 anti-*MET1* with cellularised endosperm and torpedo embryo. (F) *FIE/fie* selfed five-day-old seed with arresting embryo and uncellularised endosperm. (Bar=0.05mm.)

homozygote, but when a *FIE/fie* heterozygote was pollinated with C24 pollen, arrested and normal seeds were produced in a ratio of 1:1 (109:111) (Fig. 3.1 III A). In contrast, when pollen was taken from a C24 anti-*MET1* plant, all seeds were fully developed and viable but were in two distinct size classes in a 1:1 ratio (105:112) (Fig. 3.1 III A). The larger seeds that later proved to be derived from *fie/fis3* ovules contained normal endosperm and embryo (Fig. 3.2 E). When a *FIE/fie* heterozygote was pollinated with Columbia pollen, arrested and normal seeds were produced in a ratio of 1:1 (120:131) (Fig. 3.1 III D). In contrast, when pollen was taken from a *ddm1* plant, half the seeds were fully developed but of a small size and other half were aborted (109:115), indicating *ddm1* was not able to rescue the *fie/fis3* defect (Fig. 3.1). However, the embryos in the aborted *fie/fis3* seeds following *ddm1* pollination were arrested at the walking stick stage. In the aborted *fie/fis3* seeds following Col pollination, embryos were arrested at heart stage, indicating a partial rescue of the *fie/fis3* defect by *ddm1* pollen.

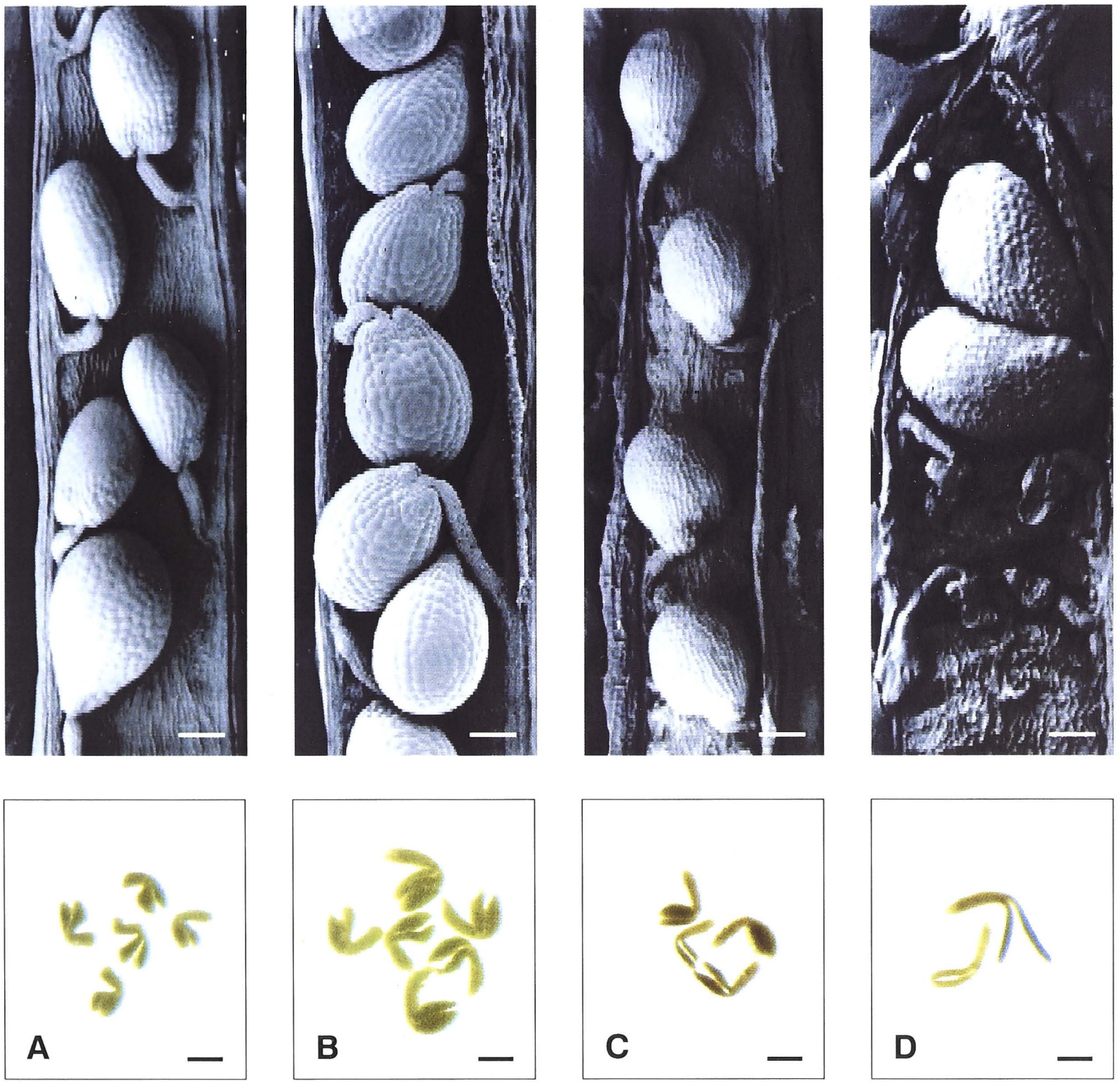
These results, where pollen from hypomethylated plants, either C24 anti-*MET1* or *ddm1,* produced normal seed in *mea/fis1, fis2,* and *fie/fis3* ovules, suggest that under low methylation conditions some gene(s) in the male genome overcome(s) the abnormalities generated in the ovules carrying mutant alleles of any of the three *FIS* loci. The hypomethylated pollen in *ddm1* was able to rescue *mea/fis1* and *fis2* maternal defect but not to completely rescue *fie/fis3*.

The above results could mean that the paternal copies of *MEA/FIS1, FIS2,* and *FIE/FIS3* normally do not function in the early development of the seed, but if they are derived from a hypomethylated male genomic environment they are reactivated and function to overcome the

deficiencies in the mutant ovules as suggested by Vielle-Calzada et al. (1999) in *mea/fis1* rescue and Vinkenoog et al. (2000) in the *fie/fis3* rescue experiment. Alternatively, the seed rescue may not require functional *MEA/FIS1*, *FIS2,* and *FIE/FIS3* genes but may result from the action of other genes, the normal pattern of activity of which is changed if DNA methylation levels are low in the pollen parent. However, no pollen-derived *MEA/FIS1*::*GUS*, *FIE/FIS3* ::*GUS* or *FIS2*::*GUS* activity was observed under conditions of low DNA-methylation (last chapter). I used the pollen from these hypomethylated *FIS* gene::*GUS* plants to rescue the maternal defect of their corresponding mutants and found that the capacity for rescuing had not been compromised by outcrossing the C24 anti-*MET1* once to the GUS reporter transgenic lines.

3.3.2 Seed size change in reciprocal crosses between L*er* and C24 anti-*MET1*.

In the last section I noted that *FIE/fie* plants pollinated with *FIE/FIE* anti-*MET1* gave rise to seeds of two size classes. To determine which size was derived from *fie/fis3* ovules and which from *FIE/FIS3* ovules, wild-type L*er* (*FIE/FIE)* was pollinated with anti-*MET1* pollen. The seeds (Fig. 3.3 A) were significantly smaller than those from selfed L*er* plants (Fig. 3.3 B) or from the reciprocal cross (C24 anti-*MET1* pollinated with L*er* pollen) (Fig. 3.3 D). Thus the smaller seeds obtained when *FIE/fie* is crossed with anti-*MET1* pollen are likely to be derived from the maternal carriers of the *FIE/FIS3* allele, and the larger seeds, from the maternal carriers of the *fie/fis3* allele. Selfed C24 anti-*MET1* plants also yielded small seeds (Fig. 3.3 C). These results show that pollen from demethylated plants reduces seed size and that this reduction is countered by the mutation in *mea/fis1, fis2,* or *fie/fis3* in the maternal genes. The



L*er* x Anti | **L*er*** | **Anti** | **Anti x L*er***

Fig. 3.3 Seed size control of hypomethylation. (A) L*er* pollinated with C24 anti-*MET1* pollen. Seeds and embryos are smaller than those in B. (B) L*er* selfed seeds and embryos. (C) C24 anti-*MET1* selfed seeds smaller than the seeds in D. (D) C24 anti-*MET1* pollinated with L*er*. Seeds become bigger than those in A. (Bars=0.05mm.)

smaller seeds gave rise to small seedlings that later grew into wild-type-sized plants.

3.3.3 Rescue of female *mea* and *fis2* by hypomethylated pollen does not require a functional paternally derived *MEA* or *FIS2* gene.

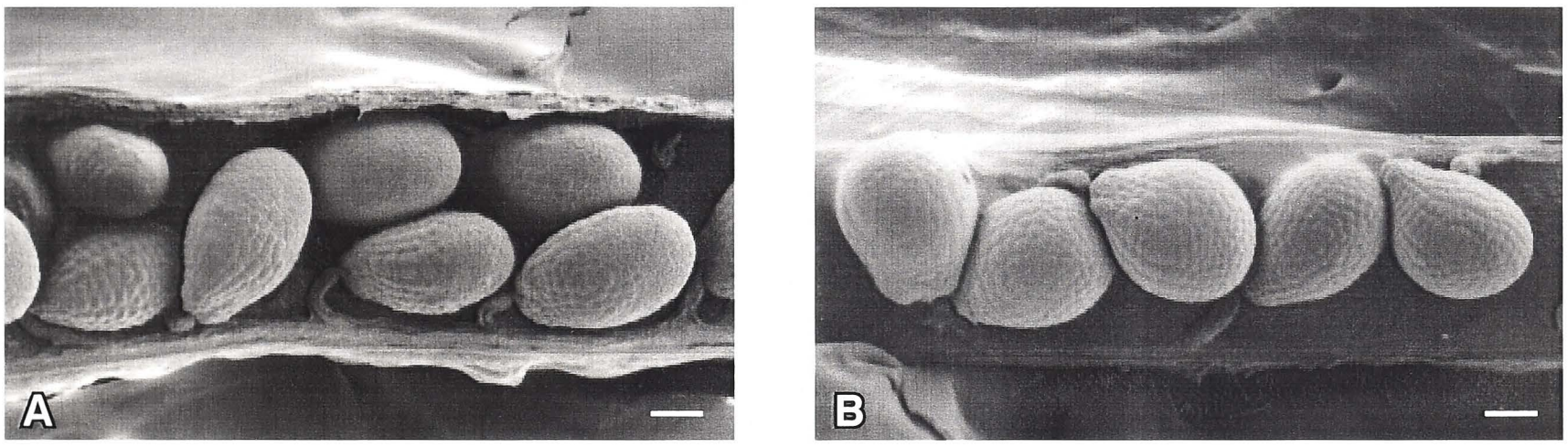
To test whether the rescue of female *mea* and *fis2* by hypomethylated pollen requires a functional paternally derived *MEA* or *FIS2* gene, *mea/fis1* and *fis2* homozygotes were pollinated by *mea/mea* anti-*MET1* or *fis2/fis2* anti-*MET1* pollen respectively, and seed rescue was observed (Fig.3.4 C). More details are shown in section 3.3.4. When *mea/mea* homozygotes were crossed with a *MEA/mea* anti-*MET1* pollen parent plant heterozygous for the anti-*MET1* construct, 248/251 seeds were rescued, in comparison to 12/130 seeds rescued in a cross with wild-type (L*er*/C24) pollen (Fig. 3.4 A). Similarly, *fis2/fis2* plants crossed with pollen from a *FIS2/fis2* anti-*MET1* plant rescued 278/298 seeds, in contrast to 1/150 rescued seeds with the wild-type (L*er*/C24) cross (Fig. 3.4 B). Because half the pollen of *MEA/mea* anti-*MET1* and *FIS2/fis2* anti-*MET1* carried the *mea/fis1* or *fis2* mutation but yet there was almost 100% rescue of the *mea/fis1* or *fis2* maternal defect, rescue of the female *mea/fis1* or *fis2* by hypomethylated pollen does not require a functional paternally derived *MEA/FIS1* or *FIS2* gene.

3.3.4 Hypomethylation rescues maternal defects of *fis* class mutants via the male but not the female genome.

3.3.4.1 Rescue of *mea/fis1* maternal defect with hypomethylated *mea/fis1* pollen.

mea/mea X
MEA/mea anti-MET1

fis2/fis2 X
FIS2/fis2 anti-MET1



mea/mea X
mea/mea anti-MET1

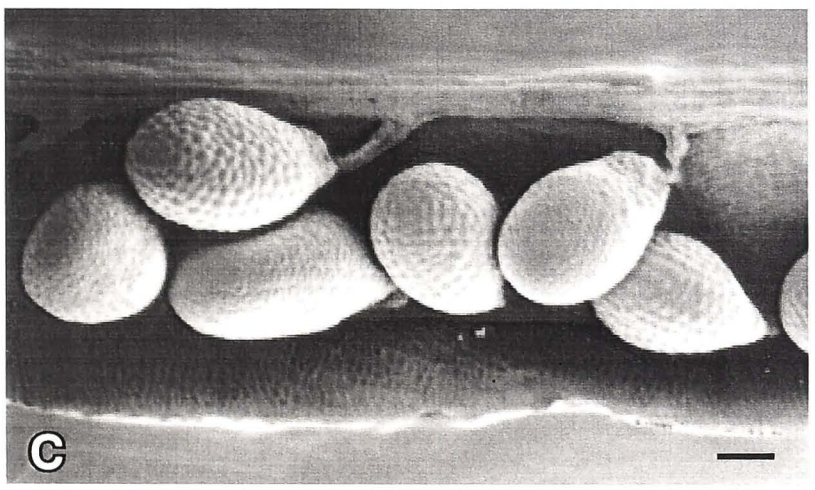


Fig. 3.4 Rescue of *fis2* and *mea/fis1* embryos by hypomethylated *fis2* or *mea/fis1* mutant pollens. (A) *mea/mea* pollinated with *MEA/mea* anti-*MET1*. (B) *fis2/fis2* pollinated with *FIS2/fis2* anti-*MET1*. (C) *mea/mea* pollinated with *mea/mea* anti-*MET1*. (Bar=0.05mm)

When pollinated with wild type L*er* pollen, a heterozygous *MEA/mea* with normal methylation level displayed 1:1 (110:109) segregation of viable to aborted seeds. Heterozygous *MEA/mea* carrying anti-*MET1*, when emasculated and pollinated with the same WT pollen, also gave 1:1 (138:123) segregation of viable to aborted seeds, indicating that the maternal hypomethylation was not able to rescue *mea/fis1* abortion (Table 1). In contrast, the pollen of *MEA/mea* anti-*MET1* was able to rescue *mea/mea* with 99.1% viable seeds (Table 1). Under selfing conditions, *MEA/mea* anti-*MET1* produced 95.5% viable seeds (Table 1).

Table 1, Rescued seed: aborted seed ratios of crosses among (*fis* class mutants x C24 anti-*MET1*)F1, L*er* and *fis* mutants

	L*er*♂	*MEA/mea*,anti ♂	*FIS2/fis2*,anti♂	*FIE/fie*,anti♂
MEA/mea anti ♀	138:123	430:20		
FIS2/fis2 anti ♀	195:180		243:15	
FIE/fie anti ♀	94:90			226:127
mea/mea ♀	108:1	147:5		
fis2/fis2 ♀	105:3		101:4	

FIE/fie ♀	157:168	98:22
MEA/mea ♀	110:109	
FIS2/fis2 ♀	83:90	

In order to extend this observation to the progeny of those crosses, I studied the rescued seeds derived from the *mea/mea* plants pollinated by the hypomethylated pollen. 17 F1 plants of *mea/mea* X *MEA/mea* anti-*MET1* were investigated for homozygosity by PCR with the *mea* mutation-detecting primers (Luo et al., 1999). The PCR fragment was cut with *Mse*I and fragments were separated on 2% agarose. Only the DNA fragment amplified from homozygous *mea/fis1* could be completely cut by *Mse*I (data not shown). Nine plants that were homozygous for *mea/mea* were obtained. One *mea/mea* anti-*MET1* was selected for further testing. After two selfing generations, one *mea/mea* anti-*MET1* plant that set approximately 90% viable seed was selected. When used as pollen donor to normally methylated *mea/mea* mutants, it could rescue about 95% seeds (Fig. 3.5 A & C). The control cross, *mea/mea* pollinated with normally methylated *mea/mea* only gave 1% viable seed. When the *mea/mea* anti-*MET1* was pollinated with *mea/mea* pollen only about 10% viable seeds were produced (Fig. 3.5 B & D), indicating that the rescue occurs only if the hypomethylated genome is introduced via pollen. Those 10 % viable seed could result from the weak modification of seed development seen in the C24 background (Luo et al., 2000).

The rescue worked with the hypomethylated pollen even when the anti-*MET1* transgene had been crossed out. I identified a *mea/mea* plant

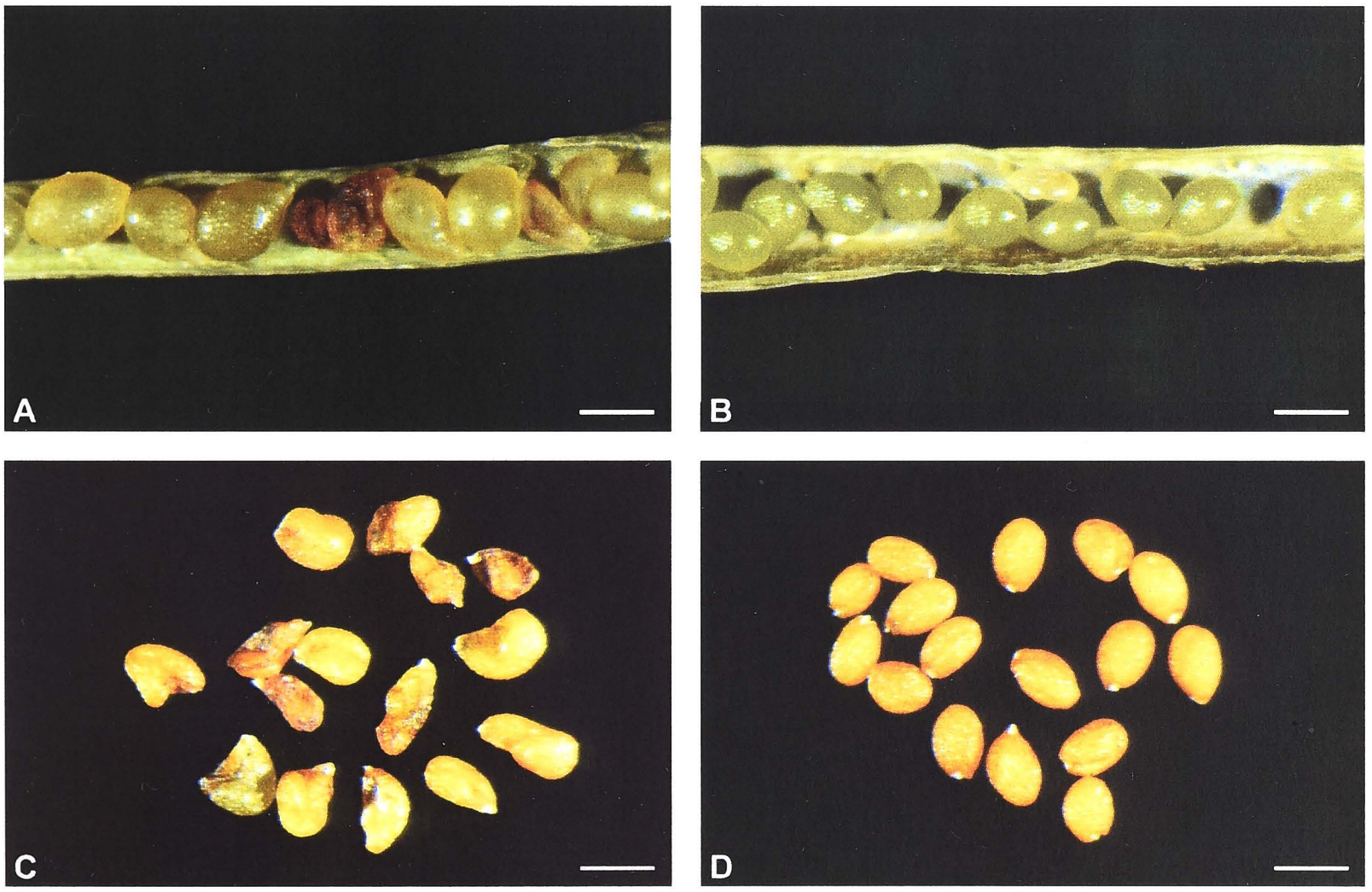


Fig. 3.5 Reciprocal crosses between *mea/mea* and *mea/mea* anti-*MET1*. (A&C) *mea/mea* anti-*MET1* pollinated with *mea/mea*, showing non-rescued seeds. (B&D*) mea/mea* pollinated with *mea/mea* anti-*MET1*, showing rescued seeds. (bar=0.05mm.)

lacking the anti-*MET1* transgene but able to rescue 95% *mea/fis1* seeds that should normally aborted, indicating that the rescue mediated by hypomethylation is independent of the presence of the transgene, anti-*MET1*. This observation also demonstrated that hypomethylation was likely to create other epi-alleles responsible for *mea/fis1* rescue.

3.3.4.2 Rescue of the *fis2* maternal defect with *fis2* hypomethylated pollen.

A *FIS2/fis2* anti–*MET1* plant, when pollinated with pollen of L*er*, displayed the same 50:50 segregation of viable to aborted seeds as a *FIS2/fis2* plant carrying no transgene was pollinated with L*er* (Table 1). In contrast, the hypomethylated pollen of *FIS2/fis2* anti-*MET1* was able to rescue the *fis2/fis2* defect and 94.2% selfed seeds of *FIS2/fis2* anti-*MET1* were viable (Table 1).

In the (*fis2/fis2* X *FIS2/fis2* anti-*MET1*) F1, homozygous *fis2/fis2* could easily be identified as plants lacking small seeds because small seeds were derived from the wild type ovules pollinated with hypomethylated pollen. One plant, confirmed by sequencing as *fis2/fis2* anti-*MET1* set viable selfed seeds (60%) and could rescue a normally methylated *fis2/fis2* plant as pollen donor with 52.5% efficiency. When pollinated with L*er*, it only gave 6.7% viable seeds. These results again indicate that hypomethylation of the maternal genome can not rescue the aborted seed phenotype.

3.3.4.3 Rescue of *fie/fis3* maternal defect with pollen from *FIE/fie* anti-*MET1*.

FIE/fie anti-*MET1* gave 50:50 (94:90) segregation of viable seeds to aborted seeds when pollinated with L*er*. Its pollen could rescue *FIE/fie* heterozygous plants to give 81.7% viable seed (table I). Even though the selfed seeds of *FIE/fie* anti-*MET1* consisted of a low propotion of viable seed (64%), the rate is still above the 50% obtained from the cross of *FIE/fie* anti-*MET1* pollinated with L*er*. I will explain later why there were more aborted seeds in the selfed *FIE/fie* anti-*MET1* than in selfed *MEA/mea* anti-*MET1*.

3.3.5 *FIE/FIS3* is essential for embryo development and exerts a sporophytic function in embryo and gametophytic one in endosperm.

I attempted to generate *fie/fie* homozygous plants by isolating the shrivelled seeds from a selfed *FIE/fie* heterozygote. Using the same method, I had easily obtained *mea/mea* and *fis2/fis2* homozygous mutant plants, indicating that the homozygous *mea/fis1* and *fis2* mutants were not embryo lethal. In support of this conclusion: there is no obvious embryo expression of *MEA/FIS1* or *FIS2*::*GUS*. I obtained about 20 plants that germinated from 1000 aborted seeds of selfed *FIE/fie* heterozygous plants. The genotypes of those aborted seeds could only be *fie* (maternal) / *fie* (paternal) or *fie* (maternal) / *FIE* (paternal). But no homozygous *fie/fie* plant was identified among them. In fact, every plant was a heterozygote *fie* (maternal) / *FIE* (paternal), indicating that the *fie/fie* embryo does not develop. Thus, both paternal and maternal *FIE* function is essential for embryo development. This conclusion is supported by the observation that maternal and paternal copies of *FIE*::*GUS* are both expressed in heart-staged embryos (Chapter 2). Therefore, *FIE* exerts a sporophytic function in embryo development but a gametophytic one in endosperm development. *FIE* was shown to be paternally imprinted in the

endosperm but not in the embryo (Chapter 2). Thus, *FIE* is an imprinted gene that has parent-of-origin expression in the endosperm but not in the embryo. Both maternal and paternal copies of *FIE* are essential for embryo development, in contrast to the lack of function of *MEA/FIS1* or *FIS2* in embryo development.

I also tried to obtain homozygous *fie/fie* anti-*MET1* plants from a rescued population. When *FIE/fie* was pollinated with *FIE/fie* anti-*MET1*, 81.7% of the seeds were rescued (Table I). However I could not identify any homozygous *fie* (maternal) / *fie* (paternal) plants in the progeny, indicating that the hypomethylation could not rescue the *fie/fie* embryo lethality. Those seeds in the *FIE/fie* plants pollinated with *FIE/fie* anti-*MET1* pollens which were not rescued could have been *fie/fie* homozygotes, which should account for 25 % of total seeds. 19.3% (100%-81.7%) aborted seeds were observed in above cross. Thus the rescue of the *fie/fis3* defect is different from that of the *mea/fis1* or *fis2* defect because *fis2/fis2* and *mea/mea* can be rescued with anti-*MET1* pollen carrying *fis2* or *mea* mutant genes. In contrast to *fie/fie, fis2/fis2* or *mea/mea* are not embryo lethal. Therefore, there are two components involved in the *fie/fis3* defect rescue: the rescue of embryo and the rescue of endosperm. Because of the sporophytic function of *FIE* in the embryo, the paternal genome needs to carry a *FIE* copy for embryo rescue. But it is difficult to test if paternal *FIE* function is necessary for endosperm rescue. The *fie/fie* embryo is aborted, even if the endosperm may have been rescued. The *fie/fie* embryo lethality also explains why there were more aborted seeds in the selfed *FIE/fie* anti-*MET1* than those in the selfed *mea/mea* anti-*MET1*. A paternal copy of *FIE* must be provided in order to rescue the maternal embryo defect in ovules carrying a *fie* mutation, because the *fie/fie* embryo is lethal without a paternal *FIE* copy.

This result is consistent with the observation made by Vinkenoog et al. (2000), who did a similar experiment. However, their interpretations about the results were different. Because they did not observe the lethality of *fie/fie* embryos, Vinkenoog et al. (2000) concluded that the rescue of *fie/fis3* maternal defect mediated by hypomethylation needed the reactivation of *FIE/FIS3* copy in the male genome. I believe that, because the embryo needs a sporophytic function of *FIE/FIS3* for proper growth, the conclusion that the paternal copy of *FIE/FIS3* is reactivated under hypomehtylation based on the genetic experiment by Vinkenoog et al. 2000 is not warranted.

In support of this conclusion, I have shown that there is no paternal GUS reactivation in endosperm in the *FIE/FIS3::GUS* transgenic plants with low levels of DNA methylation.

3.3.6 The chromosomal locations of putative paternal modifiers for *fis* genes.

Since the rescue of *fis2* and *mea/fis1* mutant seeds by paternal hypomethylated pollen does not involve the reactivation of *FIS2* and *MEA/FIS1*, or possibly *FIE/FIS3*, there must be other genes the paternal copies of which are affected by hypomethylation and which cause the rescue of the *fis* maternal defect. But what are these genes and how many of them are there?

In order to map these *Paternal Modifiers of FIS* (*PMF*), I crossed a C24 anti-*MET*I that was heterozygous for the transgene but able to completely rescue both *fis*2 and *mea/fis1* seeds, to L*er*. In the F1 population, half the plants had no anti-*MET1* transgene. The F1 plants with no transgene were

crossed as pollen donor to *mea/mea* and *fis2/fis2* mutants. The proportion of rescued seeds ranged from 30% to 50%. These results showed that an F1 pollen donor carrying no anti-*MET1* transgene was able to recue *fis2* and *mea* seeds, indicating that the rescue did not need the presence of the transgene to maintain the epigenetic marks made by hypomethylation on the putative *PMF*s. A range of sslp markers distinguishing the C24 and L*er* genomes were used to test the population derived from the rescued seeds. If there are epigenetic *PMF* alleles made by hypomethylation in the C24 genome, then the rescued seeds must carry those *PMF* alleles. So the linked sslp markers of *PMF* alleles will show a distorted segregation ratio rather than a 1:1 ratio. Table 2 summarised the genetic analysis data.

Table 2, Genetic mapping of Paternal Modifiers of *FIS* genes (*PMF*s)

Marker	*fis2*x(L*er*xc24 anti)	*mea*x(L*er*xc24 anti)	*pi2*x(L*er*xc24 anti)
Nga63 I	10H :8L		
Nga111 I	43H:20L	15H:17L	
Nga 361 II	40H:16L	22H:8L	13H:19L
AthBio2 II	12H:9L		
Nga172 III	13H:8L	14H:16L	15H:16L
F3996FR III	15H:18L		
Nga6 III	44H:23L	44H:21L	18H:14L
Nga151 V	10H:12L		

H: Heterozygote; L: L*er*

I tested sslp markers on 4 different chromosomes. In both crosses, *fis2*x(L*er*xC24 anti-*MET1*)F1 and *mea*x(L*er*xC24 anti-*MET1*)F1, markers nga6 and nga 361 showing polymorphism between L*er* and C24 did not segregate in a 1:1 ratio in the rescued seed population, while they did

segregate in a 1:1 ratio in the control population, L*er*x(L*er*xC24 anti *MET1*)F1. These results suggested that these markers were linked to two independent *PMF*s that were responsible for rescuing both *fis2* and *mea/fis1* defects. Nga 111 segregated 1:1 (15:17) in the *mea/fis1* rescued seed population but not in the *fis2* rescued seed population (43:20), suggesting that there may be a different locus specific to *fis2* rescue. There may be different *PMF*s for *fis2* and *mea* rescue. Other markers tested showed no variation in segregation from the 1:1 ratio. These genetic data support the suggestion that multiple loci are modified by hypomethylation that are responsible for the rescue. The nature of these epigenetic alleles remains to be identified.

3.4 Discussion

3.4.1 Rescue of female *mea/fis1*, *fis2* and possibly *fie/fis3* endosperm defect by hypomethylated pollen does not require a functional paternally derived *MEA*, *FIS2* or *FIE* but hypomethylated paternal genome.

Vielle-Calzada et al. (1999) suggest that the rescue of the arrested-seed phenotype is caused by the reactivation of the paternally derived wild-type *MEA/FIS1* allele in the developing seed, the reactivation being dependent on the reduced methylation level or on chromatin restructuring caused by the *ddm1* allele. Vinkenoog et al. (2000) concluded that the rescue of *fie/fis3* maternal defect mediated by hypomethylation needed the reactivation of the *FIE/FIS3* copy in the male genome. However the lack of reactivation of paternal copies of the various *FIS*::*GUS* construct under hypomehtylation condition and the capacity of hypomethylated *fis2* (or *mea/fis1*) mutant pollens to rescue the *fis2* (or *mea/fis1*) defect did not

support the above conclusion. In the experiment by Vielle-Calzada et al. (1999), *ddm1* is in a Col background. A strong modifier or modifiers exist in the Columbia background (data not shown.), which could result in *mea/mea* or *fis2/fis2* mutants setting a high proportion of viable seeds in the Col background. The modification in the Col background could have interfered with the genetic analysis made by Vielle-Calzada et al. (1999). My results indicate that the reactivation of paternally derived *MEA/FIS1* and *FIS2* genes cannot be responsible for the seed rescue I observed in the *mea/fis1* and *fis2* ovules. Thus hypomethylation must alter the activity of other, as-yet-unidentified, genes derived from the pollen that have a role in the early developing seed. These results are in agreement with the previous observation that the paternal imprinting of *MEA/FIS1* and *FIS2* is not altered by hypomethylated pollen.

In the case of *fie/fis3* rescue, because of the sporophytic function of *FIE/fis3* in the embryo (no such a function has been observed for *FIS1/MEA* and *FIS2*), the rescue of the *fie/fis3* defect is different from that of *mea/fis1* or *fis2* defect. In contrast to *fie/fie*, *fis2/fis2* or *mea/mea* mutation are not lethal to the embryo. Therefore, there are two components involved in the *fie/fis3* rescue: the rescue of the embryo and the rescue of endosperm. The paternal *FIE/FIS3* function is essential for the embryo rescue and may not be necessary for the endosperm rescue. This is consistent with the result that the paternal imprinting of *FIE/FIS3* was not reactivated by the hypomethylation of pollen (Chapter 2). The rescue of the *fie/fis3* endosperm could be mediated by the same mechanism as the *mea/fis1* and *fis2* rescue.

The seeds of reciprocal crosses between *mea/mea* X *mea/mea* anti-*MET1* produced different seed phenotypes. *mea/mea* pollinated with *mea/mea*

anti-*MET1* produce viable wild type looking seeds, while *mea/mea* anti-*MET1* produced almost all aborted seeds when pollinated with *mea/fis1* or L*er* pollen, supporting the conclusion that hypomethylation has a parent of origin effect in *mea/fis1* defect rescue. Thus hypomethylation is essential to produce an epi-modified male genome to rescue the maternal *mea/fis1* defect. The same is true for *fis*2 rescue by hypomethylation. Genetic mapping showed that there are at least two *PMF* loci for *fis1/mea* and three for *fis2* that were modified by DNA hypomethylation. It is possible that only the paternal copies of the modifiers are altered by the hypomethylation and result in the rescue of the *fis* maternal defect when introduced from a paternal hypomethylated genome. The nature of these genes needs to be identified.

3.4.2 Hypomethylation together with *FIS* genes mediates parent of origin effects on seed development by regulating downstream genes.

Each of the *FIS* genes shows a parent-of-origin effect; paternally derived wild-type genes are not able to rescue the maternally derived lesion in the corresponding gene (Chaudhury et al., 1997; Ohad et al., 1997). Scott et al. (1998) presented a cytological investigation of seed development following interploidy crosses in *Arabidopsis thaliana*. Crosses between diploid and tetraploid plants in either direction, resulting in double the normal dose of maternal or paternal genomes in the seed, produce viable seeds containing triploid embryos. The phenotypes of (4x X 2x) and (2x X 4x) can be explained if female and male gametes contribute different sets of active alleles to the seed. In this chapter the reciprocal crosses between L*er* wild type and C24 anti-*MET1* were shown to produce seed of different sizes. The hypomethylation crosses closely phenocopy the effects of interploidy crosses. The same results were observed by Adams

et al. (2000). L*er* pollinated with C24 anti-*MET1* pollen produced smaller seeds with lower endosperm content, which resemble the seeds observed in a tetraploid plant pollinated with diploid pollen and phenocopying an excess of maternal genome. On another hand, C24 anti-*MET1* pollinated with L*er* pollen produced bigger seeds with delayed endosperm cellularisation and greater endosperm content (Adams et al., 2000), which resembled the strong paternal excess phenotype observed in diploid plants pollinated with tetraploid pollen.

To explain the above results, Adams et al. 2000 proposed a model for the action of DNA methylation in parental imprinting in plants. Global DNA hypomethylation appears to activate maternal copies of genes that would normally be active only in the male genome (in sperm), and activate paternal copies of genes that would normally be active only in the female genome (in the central cell neuleus). Thus hypomethylation has the effect of 'paternalizing' the female genome and 'maternalizing' the male genome. Based on this model, then the maternally active *FIS* genes would be de-repressed in the paternal genome. However my results did not show any change in *FIS* genes::*GUS* expression in the hypomethylated lines. Moreover, the rescue of the female *mea/fis1*, *fis2* and possibly *fie/fis3* defect by hypomethylated pollen does not require a functional paternally derived copy of *MEA/FIS1*, *FIS2* or *FIE/FIS3* but rather a hypomethylated paternal genome, suggesting that hypomethylation of genes other than *FIS* is responsible for rescuing the *fis* maternal defect. The genetic mapping described in this chapter showed that there are at least two *PMF* loci for *fis1/mea* and three for *fis2*, which were modified by DNA hypomethylation and responsible for rescuing the *fis* defect. Recently Kohler et al (2003) showed that the MADS-box gene *PHERES1* (*PHE1*) is deregulated in the *fis*-class mutants. Both *MEA/FIS1* and

FIE/FIS3 directly associate with the promoter region of *PHE1*, suggesting that *PHE1* expression is epigenetically regulated by PcG proteins. Reduced expression levels of *PHE1* in *mea/fis1* mutant seeds can suppress *mea/fis1* seed abortion, indicating a key role of *PHE1* repression in seed development. *PHE1* expression in a hypomethylated *fis1/mea* mutant background resembles the wild-type expression pattern and is associated with rescue of the *fis1/mea* seed-abortion phenotype. One *PMF* locus, which rescues the *fis2* phenotype, maps close to the location of *PHE1* on chromosome one, indicating *PHE1* could be one of the *PMFs*. *PHE1* and other *PMF*s are regulated by both *FIS* genes and DNA methylation and are involved in mediating the parent of origin effect in seed development.

CHAPTER 4

MINISEED3 (MIN3), a *WRKY* family gene, is a regulator of endosperm development and seed size in *Arabidopsis*

4.1 Introduction:

Angiosperm seed development is initiated following the double fertilization of the egg and the central cell of the embryo sac. This leads to the formation of a diploid embryo and triploid endosperm in the developing seed (reviewed by Chaudhury et al., 2001). Endosperm development progresses through several phases: syncytial growth, cellularisation, and cell death. The syncytial phase is formed by successive divisions of the triploid nuclei without cytokinesis (Boisnard-Lorig et al., 2001) and has cytoplasm which is initially compartmentalized into nuclear cytoplasmic domains (NCDs) (Olsen et al., 2001). Subsequent cellularisation, which begins in the region surrounding the embryo and proceeds towards the chalazal region (Olsen et al., 2001), converts the NCDs into endosperm cells. A cyst-like structure of free nuclear endosperm remains at the chalazal region of the embryo sac. The cell biology of endosperm cell division and maturation (Olsen et al., 2001, Boisnard-Lorig et al., 2001) is now being studied at the level of gene action. Some genes have mutant alleles which result in changes to endosperm development and have a gametophytic mode of action. Other loci operate in sporophytic mode, and when homozygous for mutant alleles, also result in modified endosperm development and a small seed size phenotype.

Additionally epigenetic controls operate in the determination of seed size. In lines with reduced DNA methylation, the parent-of-origin of the hypomethylated genome affects progeny seed size (Luo et al., 2000; Adams et al., 2000). A paternal genome with reduced DNA methylation reduces seed size, while a maternal genome with reduced DNA methylation increases seed size.

Garcia et al. (2003) described two mutants in *Arabidopsis*, *haiku1 (iku1)* and *haiku (iku2)*. These mutants are recessive, show a sporophytic mode of action, and cause premature arrest of endosperm growth and precocious cellularisation resulting in reduced seed size. These genes have not been isolated. In the present study we screened for mutants with reduced seed size and identified two loci, *MIN2* and *MIN3*, with mutant alleles, *min2* and *min3*, which reduce seed size and show developmental properties in relation to endosperm and seed development similar to the *iku* mutants. *min3* maps to a location different to either of the *iku* loci but *min2* may well be an allele of *iku2*.

The inference from our work is that the wild-type alleles of *MIN2* and *MIN3* are required for normal endosperm development. *MIN3* encodes a WRKY-type transcription factor which would have a role in the regulation of genes controlling endosperm development. The *WRKY* gene appears to negatively regulate its own activity, an autoregulatory feedback, although it has a positive effect on seed size, perhaps through enhancement of the activity of other genes.

4.2 Materials and Methods

4.2.1 Plant materials and growth conditions

Arabidopsis ecotypes used in this study were L*er* and Columbia. The *FIS2-2/fis2-2* heterozygote in the L*er* background was the starting material for mutagenesis (Chaudhury et al., 1997). Plants were grown under continuous artificial light at 20℃.

4.2.2 Mutagenesis and Mutant Identification

The *FIS2-2/fis2-2* heterozygote in the L*er* background was the starting material for mutagenesis (Chaudhury et al., 1997). 2 g seeds were mutagenized as described (Chaudhury et al., 1993). Each pot of the M1 plants was harvested separately. Small seeds from the M2 population were selected under a dissecting microscope and grown for further testing.

4.2.3 Microscopy

Mature seeds of L*er*, *min3* and *min2* were photographed and acquired as digital images under a dissection microscope. Developing seeds were cleared (Boisnard-Lorig et al., 2001). Specimens were examined with a Leica microscope using DIC optics or bright field. Nuclei were counted using images displayed on a video monitor attached to the microscope. The embryos of 15-day-old seeds were dissected out and photographed under the dissection microscope. The digital images were saved as tif files and processed using Adobe Photoshop 3.0.

4.2.4 DNA preparation, PCR, plant transformation

The plant DNA preparation and the PCR reaction were carried out as described by Bell and Ecker (1994).

Plants were transformed by floral dipping (Clough et al., 1998). The T1 seeds were screened with Kanamycin at 50mg/l. To verify that the transgenic plants contained the transgenes, the NPT II gene was used for amplification with the primers 5'-gaggctattcggctatga-3' and 5'-acttcgcccaatagcag-3'.

4.2.5 Genetic Mapping

To map the chromosomal location of the mutant genes, *min3* and *min2,* were crossed to the Columbia ecotype. Small F2 seeds set by the F1 plants were separated from wild type sized seeds and grown for mapping purposes. Sslp markers, on each of the 5 chromosomes, distinguishing the L*er* and Col genomes were used to test the small seed populations of each F2 plant (Bell et al., 1994). Markers showing biased segregation from the expected 1 L*er*: 2 heterozygous: 1 Col ratio were presumed to be linked to the mutant genes.

4.2.6 Detailed mapping of *MIN3*

To facilitate the molecular cloning of *MIN3*, insertion-deletion (indel) markers based on the Cereon database were designed (http://www.arabidopsis.org). To obtain an indel marker in BAC T22H22 closely linked to *min3*, a pair of PCR primers, 5'-tttatcagctcctgcatgctt-3' and 5'-agggaggctcatctccctat-3' flanking an 11 base insertion in Col at position 40067 bp in BAC T22H22 were synthesized. This newly synthesized sslp marker and nga280 (Bell et al., 1994) were used to test

545 and 765 F2 plants, respectively, derived from small seeds obtained from *min3/MIN3* L*er*/ Col F1 plants in the F2 population of *min3* crossed to Col.

More indel markers were designed to screen the recombinants between nga280 and the marker on BAC T22H22 (Fig. 4.2). *min3* was found to be located between two markers in a 43 kb region (Fig. 4.2). One of these markers is T5A14-23FR flanking a 13 base insertion at 23373bp in BAC T5A14 using primers: 5'- ttgttgtttcgtcactatccaaa-3' and 5'-tagacgttggctttggaacc-3'. Another is a CAP marker F20N2-B81FR flanking a single polymorphism site at 81136 bp in BAC F20N2 with primers: 5'-gagaagaacgaggggcaata-3' and 5'-ccctcgctctcttcaacagt-3', which gave 100bp, 34 bp and 30 bp fragments in Col, and 100bp and 64 bp in L*er* when the PCR products were digested with the restriction enzyme *Bst* NI.

4.2.7 Genetic complementation

To sub-clone the BACs, B*am* HI fragments, an E*co* RI fragment, and S*al* I fragments as marked in Fig. 4.2 were cloned into lambda EMBL4 and then recloned into the binary vector Bin 19. All the annotated genes on both BACs were obtained in the subclones except F20N2-3 which spans the interval from 12 kb to 14.4 kb on F20N2. To obtain the plasmid containing the complete F20N2-3 sequence a *Sma* I-*Sal* I fragment of F20N2 from 12 kb to 19 kb was cloned into Bin 19. The *Sal* I site was near the annotated stop codon of F20N2-3. A 500bp terminator sequence downstream of the stop site of annotated F20N2-3 was amplified by PCR and inserted into the *Sal* I site of this plasmid in the right orientation to obtain a complete sequence of the F20N2-3 gene (Fig. 4.2). The PCR

primers were 5'-ggatcatggaacgcctgtat-3' and 5'-gggggtcgacttgaatttgatttcaatacaaagtatg-3'.

The homozygous *min3* plants were transformed with the 6 sub clones made from BAC F20N2 and T5A14.

4.2.8 *AtWRKY10::GUS* construct

The annotated F20N2-3 gene corresponded to *AtWRKY10* (Eulgem et al., 2000). A *Hind* III fragment spanning from –2357 bp to +143 bp of the *AtWRKY10* gene was inserted in frame in front of a GUS reporter gene in the pBI101-2 binary plasmid. L*er* wild type was transformed with the construct. After GUS staining (DeBlock et al., 1992), ovules or flowers of the transgenic plants were cleared with lactophenol and observed with differential interference contrast microscopy.

4.2.9 DNA Sequencing and protein sequence analysis

To compare the genomic sequences between *min3* and L*er* in the *AtWRKY10* coding region, 5 sets of PCR primers were designed covering the whole coding region of *AtWRKY10*. The PCR products were sequenced with an Applied Biosystems Model 370A DNA Sequencer using fluorescent dye-labelled dideoxy terminators. Protein sequence comparison was performed using BLAST searches and multiple sequence alignments were performed with the Clustal W 1.8 program.

4.3 Results

4.3.1 Isolation of small seed mutants

Following EMS mutagenesis we detected plants which produced seeds in the M2 generation which were significantly smaller than the seed of the parental L*er*. We germinated these smaller seeds and screened the resulting plants to determine if they produced small seeds in the M3 generation. Of 500 small-seed M2 plants only two produced small seeds in the M3 and in subsequent generations. In plants homozygous for either of the two mutant alleles (*miniseed2* (*min2*), *miniseed3* (*min3*)), the seeds were significantly smaller than L*er* seeds in both weight and size (Table 1, Fig. 4.1, A, B & C).

Table 1, Seed size of L*er*, *min3* and *min2* mutants

	Weight	Dimensions (length X width)
min3	10.98 mg /1000 seeds	0.343±0.029mm x 0.225±0.022mm
min2	8.15 mg/1000 seeds	0.311mm±0.023 x 0.211±0.019mm
L*er*	20.10mg/1000 seeds	0.480±0.037mm x 0.295±0.020 mm

The mutant seeds (Table 1) were viable, germinating at a frequency similar to the L*er* controls. Seedlings grown from the *min2* and *min3* seeds were smaller than the seedlings of L*er*, presumably as a consequence of the smaller embryo (see Fig. 4.1, Ji, Jii, & Jiii). During later development the mutant plants grew to the same size as the control L*er* plants. The number of rosette leaves of *min2* and *min3* plants at flowering was similar to the number in the wild-type (Table 2). The number of seeds in each silique was comparable in the mutants and controls, as was silique length (Table 2).

Table 2, Number of rosette leaves, length of siliques, number of seeds per silique show no difference between mutants and L*er*

Number of rosette leaves at flowering	Length of siliques	Number of seeds per silique
8.06±1.30 for *min3*	10.3±0.38mm	54.1±3.25
8.15±0.90 for *min2*	9.20±0.35mm	53.55±4.30
8.2±1.38 for *L*er	9.92±0.47mm	54.1±3.25

When pollinated with wild type pollen, the F_1 seeds of both mutants were of wild type size, indicating that the mutations are recessive and sporophytic in expression. Crosses between homozygous *min3* and *min2* showed *MIN2* and *MIN3* are different loci.

4.3.2 Phenotype of small seed mutants

Cytological examination of the developing seeds of L*er*. and the two small seed mutants at 48 hours post pollination showed that the embryos of all three genotypes were at the octant stage. The mutants had a similar number of nuclei in the syncytial endosperm as L*er* (Table 3) and the developing seeds were of comparable size in all three genotypes.

At 72 hours after pollination, the seeds of the mutants were smaller than those of L*er*. At this time the endosperm of L*er* was still syncytial with 175.3±26.4 (n=34) endosperm nuclei (Fig. 4.1 D), whereas the endosperm of the mutants had cellularised, *min3* having 136.9±34.1 nuclei (Fig. 4.1 E), and *min2* having 144.5±32.5 nuclei (Fig. 4.1 F).

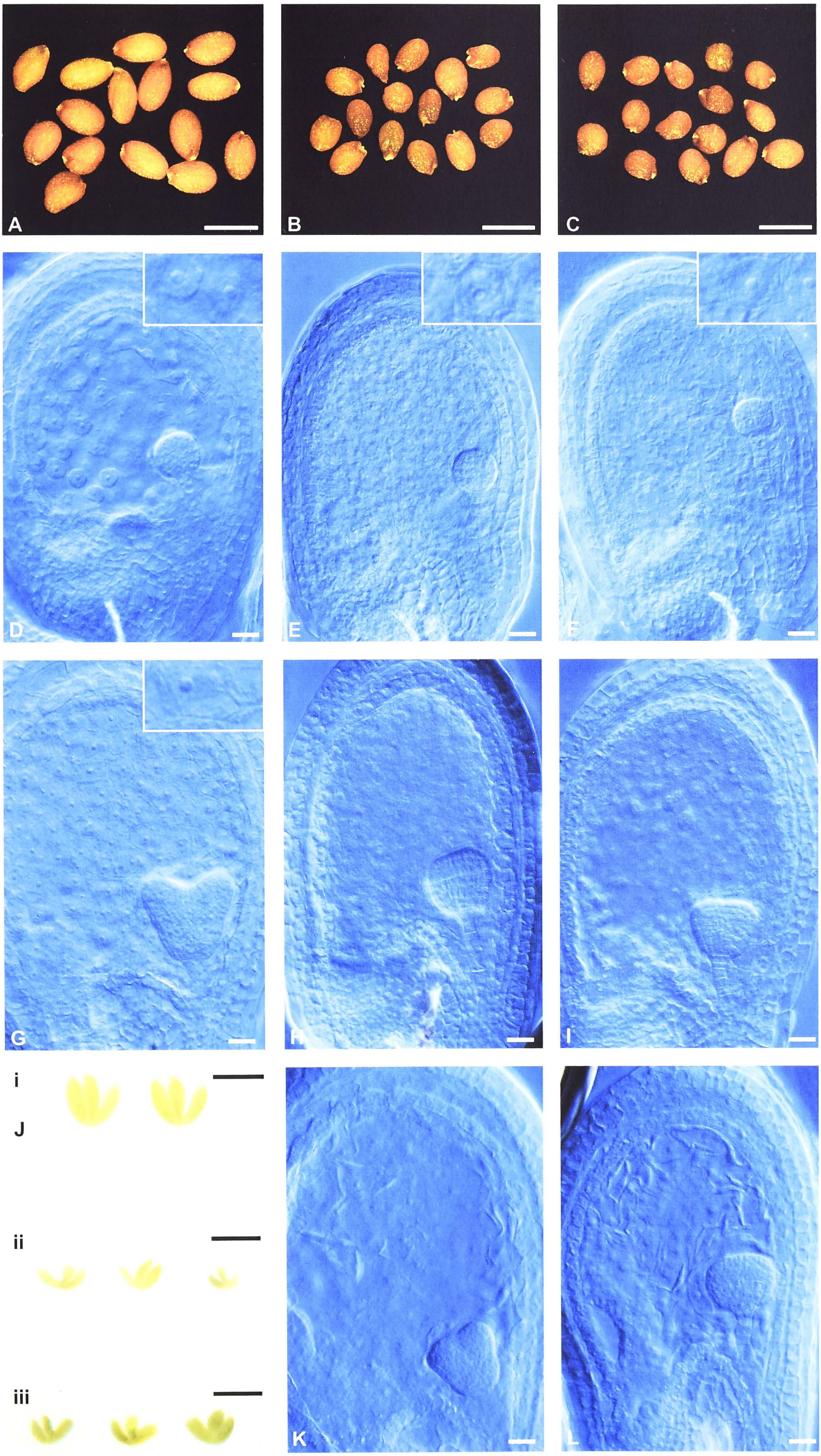

A
B
C
D
E
F
G
H
I
i
J
ii
iii
K
L

Fig. 4.1 Morphological and cytological phenotypes of *min3* and *min2*. (A) L*er* dry seeds. (B) *min3* dry seeds. (C) *min2* dry seeds. (bar=0.5mm in A, B, & C). (D) 72-hour-old wild type seed with globular staged embryo and uncellularised endosperm. Insert shows free nuclei (E) 72-hour-old *min3* seed with globular staged embryo and cellularised periphery endosperm. Insert shows cell wall of endosperm. (F) 72-hour-old *min2* seed with globular staged embryo and cellularised periphery endosperm. Insert shows cell wall of endosperm. (G) 96-hour-old wild type seed with heart staged embryo and cellularised endosperm. Insert shows cell wall of endosperm. (H) 96-hour-old *min3* seed with heart staged embryo and cellularised endosperm. (I) 96-hour-old *min2* seed with heart staged embryo and cellularised endosperm. (bar=0.05mm in D, E, F, G, H & I). (J i) 15-day-old wild type embryo. (J ii) 15-day-old *min3* embryo. (J iii) 15-day-old *min2* embryo. (bar=0.5mm in J) (K) *fis2* uncellularised endosperm (L) *fis2* and *min3* uncellularised endosperm. (bar=0.05mm in K & L.)

L*er* did not develop cellularised endosperm until 96 hours, with approximately 194.5±9.5 nuclei (Fig. 4.1 G). At 96 hours post pollination the seed size of the mutants had not increased over the size at 72 hours but in L*er* the seeds were considerably larger than at 72 hours. Embryo development was slower in the mutants; in L*er* the embryos had reached the mid-heart stage whereas in both mutants the embryos were at an early-heart stage (Fig. 4.1 G, H & I). There was no difference in cell size between the embryos of the mutants and wild type.

At 120 hours post pollination, L*er* embryos were either at the late-heart or torpedo stage of development and the endosperm was fully cellularised with 409.3±35.5 (n=10) cells, whereas, *min2* and *min3* had only 250.9 ± 52.5 (n=8) and 168.9± 39.3 (n=8) endosperm cells respectively. The embryos of the mutant seeds were at the late-heart stage of development but were considerably smaller than the wild-type embryos (Figs not shown).

Table 3, Endosperm and embryo phenotypes of the mutants

	48h	72h	96h	120h
L*er*	52.8±10.2(n=12) syncytial en octant em	175.3±26.4(n=34) syncytial en globular em	194.5±9.5 (n=9) cellularised en heart em	409.3±35.5 (n=10)
min3	48.1±3.8(n=9) syncytial en octant em	136.9±34.1 (n=11) cellularised en globular em	154.9±34.3 (n=17) cellularised en early heart em	250.9±52.5 (n=8)
min2	42.3±7.8(n=9) syncytial en octant em	144.5±32.5 (n=11) cellularised en globular em	153.4±37.9 (n=5) cellularised en early heart em	168.9±39.3 (n=8)

At 15 days post fertilization, the mature embryos of both mutants were significantly smaller than that of L*er* (Fig. 4.1, Ji, Jii, & Jiii).

4.3.3 Map location of the small-seed mutants

The locations of the small-seed mutations were mapped in crosses with the Columbia ecotype. The marker nga280 is closely linked to *min3* on chromosome 1. Among 765 F2 plants (1530 chromosomes) derived from small seeds obtained from *min3/MIN3* L*er*/ Col F1 plants in the F2 population, only five plants showed heterozygous genotype for this marker and others were L*er* genotype. Similarly with the marker nga162 on chromsome III, five heterozygotes and 49 L*er* homozygotes were detected among 54 plants derived from small seeds obtained from *min2/MIN2* L*er*/ Col F1 plants in the F2 population, indicating *min2* is close to the marker nga162 on chromosome 3. In two other mutants with small seed size, *iku1* and *iku2* (Garcia et al., 2003), which had been isolated on the basis of precocious endosperm cellularisation , the number of endosperm nuclei, the time of endosperm cellularisation and the size of embryos and seeds through development were similar to these traits in *min2* and *min3*. *iku2* is in a similar chromosomal position to *min2* on chromosome 3. *iku1* is located on chromosome 2 and thus does not correspond to *min3*. Garcia et al (2003) isolated two other alleles of i*ku1* and a further allele of *iku2;* these additional alleles had the same phenotypes as the original mutants.

4.3.4 *min3* and *min2* mutations did not rescue the *fis2* maternal abortion defect and produced small seeds in the *fis2* background.

We had earlier demonstated that *fis2/fis2* mutants, involved in endosperm development, produced aborted seeds when pollinated with wild type pollen (Chaudhury et al., 1997); in these seeds endosperm overproliferated with an increased nucleus number, remaining uncellularised. However *fis2/fis2* homozygotes produced viable, wild type-sized seeds if pollinated with hypomethylated pollen (Luo et al., 2000; Finnegan et al., 1996). In these seeds the endosperm had a normal number of nuclei and cellularised at the expected time.

Since *min2* and *min3* showed precocious cellularisation of the endosperm, we tested for the ability of the *min2* and *min3* mutations to rescue the gametophytically determined *fis2* seed abortion phenotype. In *min3/min3, FIS2/fis2* plants, a 1:1 ratio of viable to shriveled seeds was observed. Approximately 50% of seeds contained cellularised endosperm and were viable while the other 50% of seeds contained uncellularised endosperm and aborted indicating that *min3,* a sporophytically operating allele could not rescue the *fis2* abortion phenotype (Fig. 4.1 K & L).

Seed derived from *fis2* ovules of homozygous *min3/min3* plants were smaller than those derived from *fis2* ovules of *MIN3* plants. This shows that *min3* reduces the size of *fis2* seed without being able to rescue the seed abortion phenotype.

In crosses involving *min2* and heterozygous *fis2,* 49% of seed aborted (153/310) and the *fis2* ovules also produced smaller seeds in the *min2/min2* background than in *MIN2/MIN2*. Garcia et al (2003) observed a similar lack of interaction between *fis1* and the *iku* mutants. There is no evidence of any direct interaction between either of the gametophytically operating *FIS* loci and the sporophytically operating *MIN* loci.

4.3.5 Cloning of the *MIN*3 locus

*MIN*3 maps to a 43kb region from chromosome 1 between the markers T5A14-23FR and F2ON2-B81FR on the BACs T5A14 and F2ON2 (Fig. 4.2). Six overlapping segments covering this 43kb interval were introduced into *min3/min3* mutant plants and 10-20 transformed plants were obtained for each segment. Among the 6 transformations, only *min3/min3* plants infected with Agrobacterium carrying F20N2-3, produced up to 1% big seeds among T1 seeds (Fig. 4.3 A). When stable transformants were selected, large T2 seeds in all siliques were produced in 16 plants, which all carried the F20N2-3 segment (Fig. 4.3 Bii). Mutant and wild type developing seeds at a comparable stage are shown in Fig. 4.3 Bi and Biii.

The F2ON2-3 plasmid encodes a single gene corresponding to *AtWRKY10*, a member of the WRKY family of transcription factors (Fig. 4.4) (Eulgem et al., 2000). The predicted amino acid sequence of ATWRKY10 contained a WRKY domain located from amino acid residue 301 to amino acid residue 370 (Fig. 4.4 B). The WRKY domain is defined by the conserved amino acid sequence WRKYGQK at its N-terminal end, together with a novel zinc-finger-like motif C-X4-5-C-X22-23-H-X1-H (Fig. 4.4 B). The coding region of *AtWRKY10* in the *min3* mutant show a single nucleotide substitution (G to A) in the zinc finger motif of the conserved WRKY domain compared to that of L*er*. The nucleotide change is predicted to result in the conversion of a glutamic acid to a lysine residue. We identified a full length *AtWRKY10* cDNA from GenBank (accession number: AY071851). The genomic sequence (AT1G55600.1) contains 5 exons and 4 introns (Fig. 4.4 A). No function has been assigned to the *WRKY10* gene.

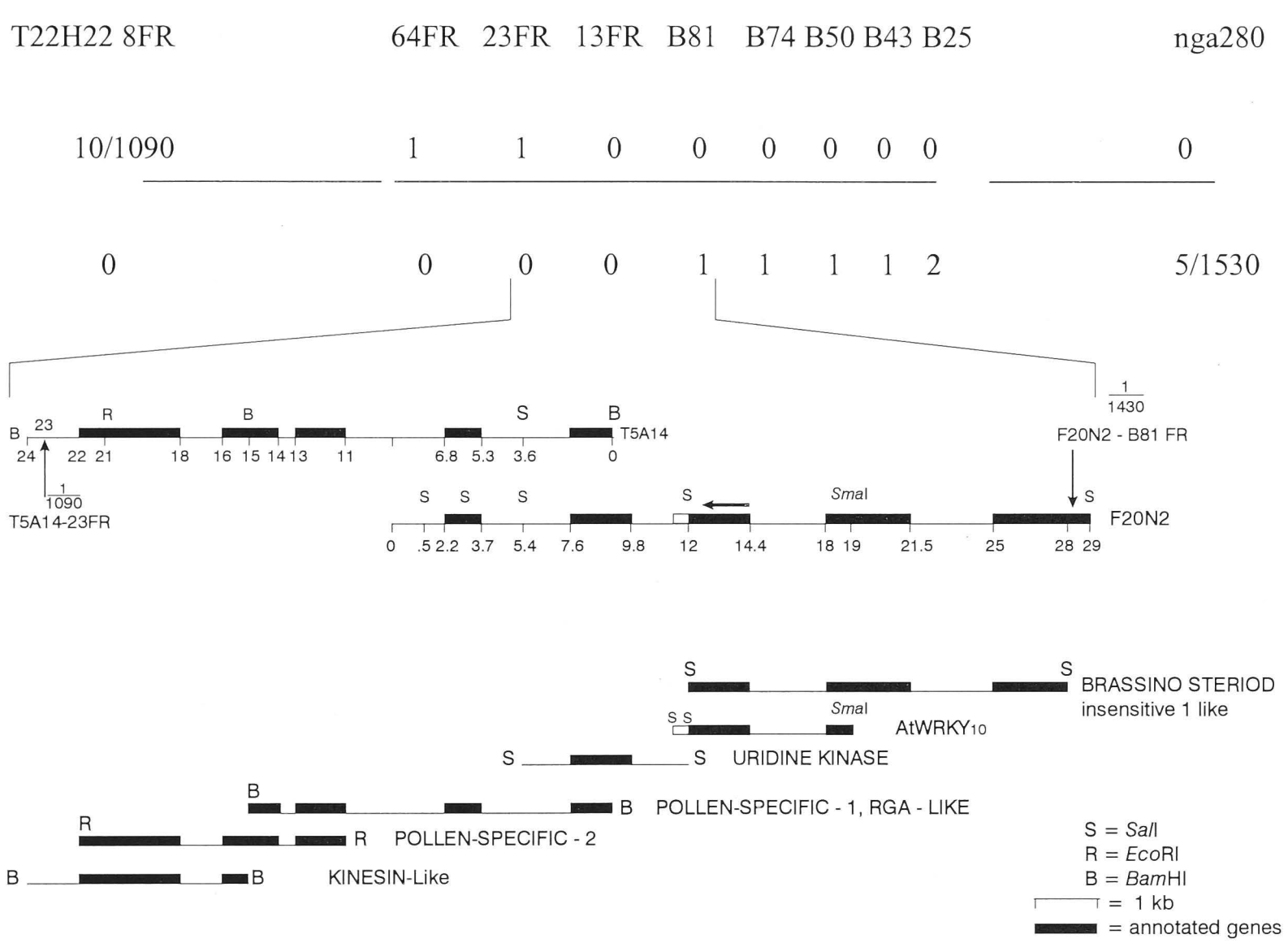


Fig. 4.2. Positional cloning of *MIN3* gene. 10 PCR based markers were used to screen 765 *min3* mutant plants which represent 1530 chromosomes in an F2 population of *min3* crossed with Columbia. The numbers of heterozygous plants detected with each marker were indicated. The *min3* was located between markers T5A14 23FR and F20N2 B81. The restriction sites and annotated genes were marked on both Bacs. The subcloned fragments in a binary vector were also indicated.

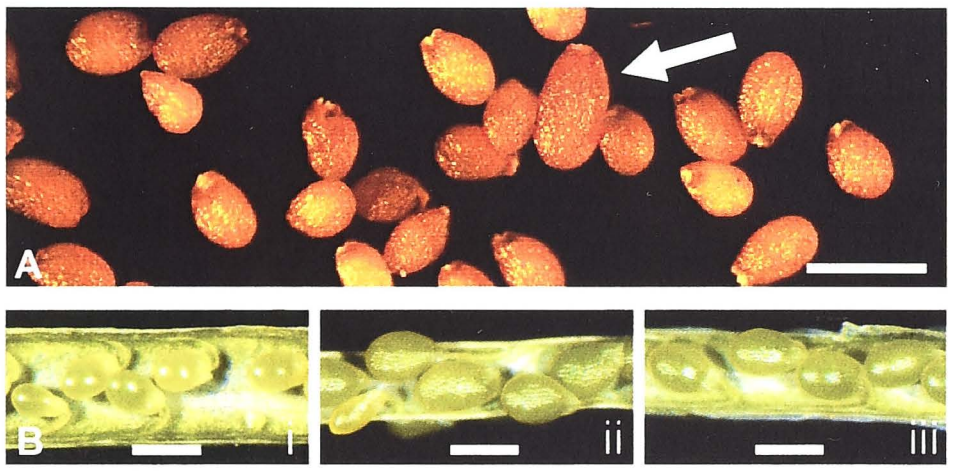


Fig. 4.3. Complementation of *min3* mutant phenotype. (A) Transient endosperm complementation. *min3* mutant dipped with *AtWRKY10* plamsid produced wild type sized seeds, arrows indicated. (B i) *min3/min3* untransformed silique, showing only small seeds. (B ii) *min3/min3* silique transformed with *AtWRKY10*, showing big seeds and one small seed. (B iii) L*er* seeds. (bar=0.5mm)

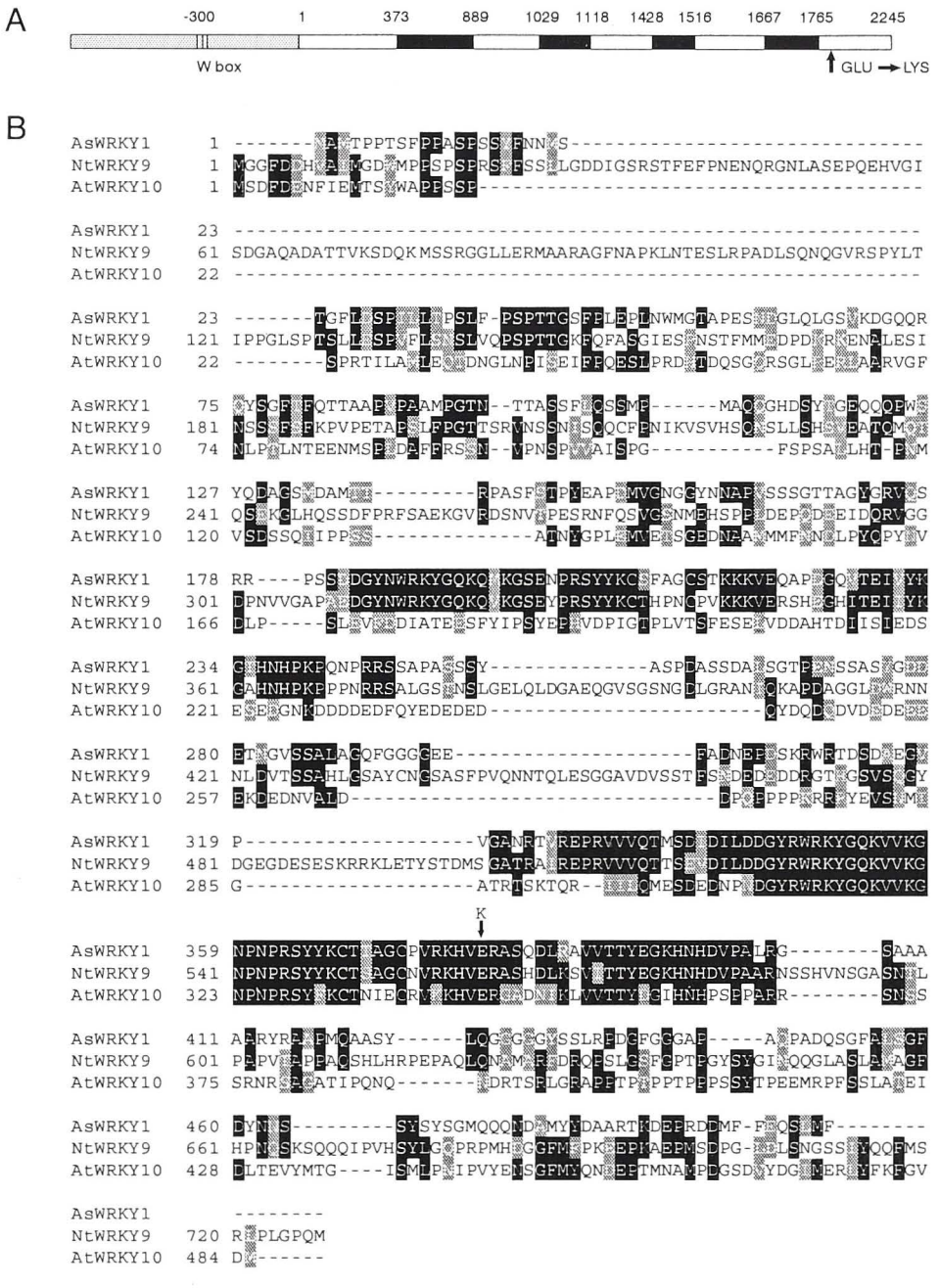


Fig. 4.4. ***MINIS3/AtWRKY10*** **gene structure and sequence.** (A) Positions of exon, intron and mutation in *min3*. Black and open boxes represent exons and introns, respectively. (B) Comparison of the predicted AtWRKY10 polypeptide to proteins with WRKY domains. Optimal alignment of amino acid sequences was determined by using the **ClustalW 1.8** method. WRKY domains of AtWRKY10 are from residue 301 to 370. Arrow shows the mutation in *min3,* a substitution of K from E.

WRKY proteins bind to W box motifs which have a consensus sequence (T)TGAC(C/T). This motif is present in the promoter of many plant genes associated with defense (Ruthton et al., 1998). We identified three W-box like elements within 300 bp of the translation start in the promoter of the *MIN3 (WRKY10)* gene (Fig.4 A). One W box motif has the sequence TTGACC, identical to the consensus sequence motif; the other two motifs ATGACG and CTGACA, contain the four core bases. The presence of the W-box motifs in the promoter of *WRKY10* suggests it may be autoregulated – a characteristic shown by several other *WRKY* genes (Robatzek & Somssich., 2002; Chen & Chen., 2002).

4.3.6 *MIN3/AtWRKY10::GUS* is expressed in developing seeds and pollen

An AtWRKY10::GUS translational fusion was introduced into L*er*. *GUS* expression was found in the 12 to 96-hour -old developing seeds following self-pollination of these plants (Fig. 4.5 B, C, D & E) but not in unfertilized ovules. Two to eight free-endosperm nucleate stages in the 12 and 24-hour-old seeds showed *GUS* expression (Fig. 4.5 B & C). 72 or 96-hour-old seeds had *GUS* expression in both the embryo of the globular stage and in the endosperm (Fig. 4.5 D & E), with high expression in the Embryo Surrounding Region (ESR) (Fig. 4.5 D & E). Expression was not seen in the 110-hour-old seed with a full heart stage embryo.

The unfertilized central cell nucleus and egg did not show any GUS expression. All pollen grains were stained blue in a homozygous *AtWRKY10::GUS* plant (Fig. 4.5 A i), while a hemizygous *AtWRKY10::GUS* plant produced approximately 40% blue pollen indicating that *MIN3* has male gametophytic expression. GUS

expression was not seen in any other part of the plant indicating the *AtWRKY10* gene may function solely in the developing seeds and pollen. When a plant carrying *AtWRKY10::GUS* was pollinated with wild type, the same expression pattern in endosperm and embryo was observed.

To follow early seed expression from a paternally derived *AtWRKY10::GUS* construct a L*er* plant was pollinated with At*WRKY10::GUS* pollen and the ovules checked for *GUS* expression at 12, 16, 24, 48, 72, 96 and 110 hours post fertilization. The germinating pollen on the L*er* stigma showed GUS activity in the pollen tube (Fig. 4.5 A ii). In the fertilized ovules, endosperm nuclei showed GUS staining as early as 12- hours after fertilization at the two free nucleate stage (Fig. 4.5 F). In 24 to 96 hour old seeds, *GUS* expression is the same as that in the selfed or maternally derived ovules. The 110-hour-old seed with full heart stage embryo did not show *GUS* expression similar to the situation when the GUS gene was provided by the female plant. Thus both the paternally and maternally derived *AtWRKY10:GUS* gene is active in the developing endosperm at early stages and in the embryo. The gene showed no expression in the unfertilized embryo sac.

4.3.7 *MIN3* is an autorepressive gene

A *MIN3/MIN3 AtWRKY10::GUS* plant was pollinated with either L*er* or *min3* pollen. The ovules were stained to check GUS activity at 48 and 72 hours post pollination. GUS staining in the ovules pollinated with *MIN3* (*MIN3/MIN3/MIN3* endosperm) was of lower intensity than that in the ovules that are *MIN3/MIN3/min3* (Fig. 4.5 G & H) endosperm. About 20 comparisons of ovules made in this experiment showed the same higher intensity of GUS staining in the endosperm carrying a mutant allele. In a

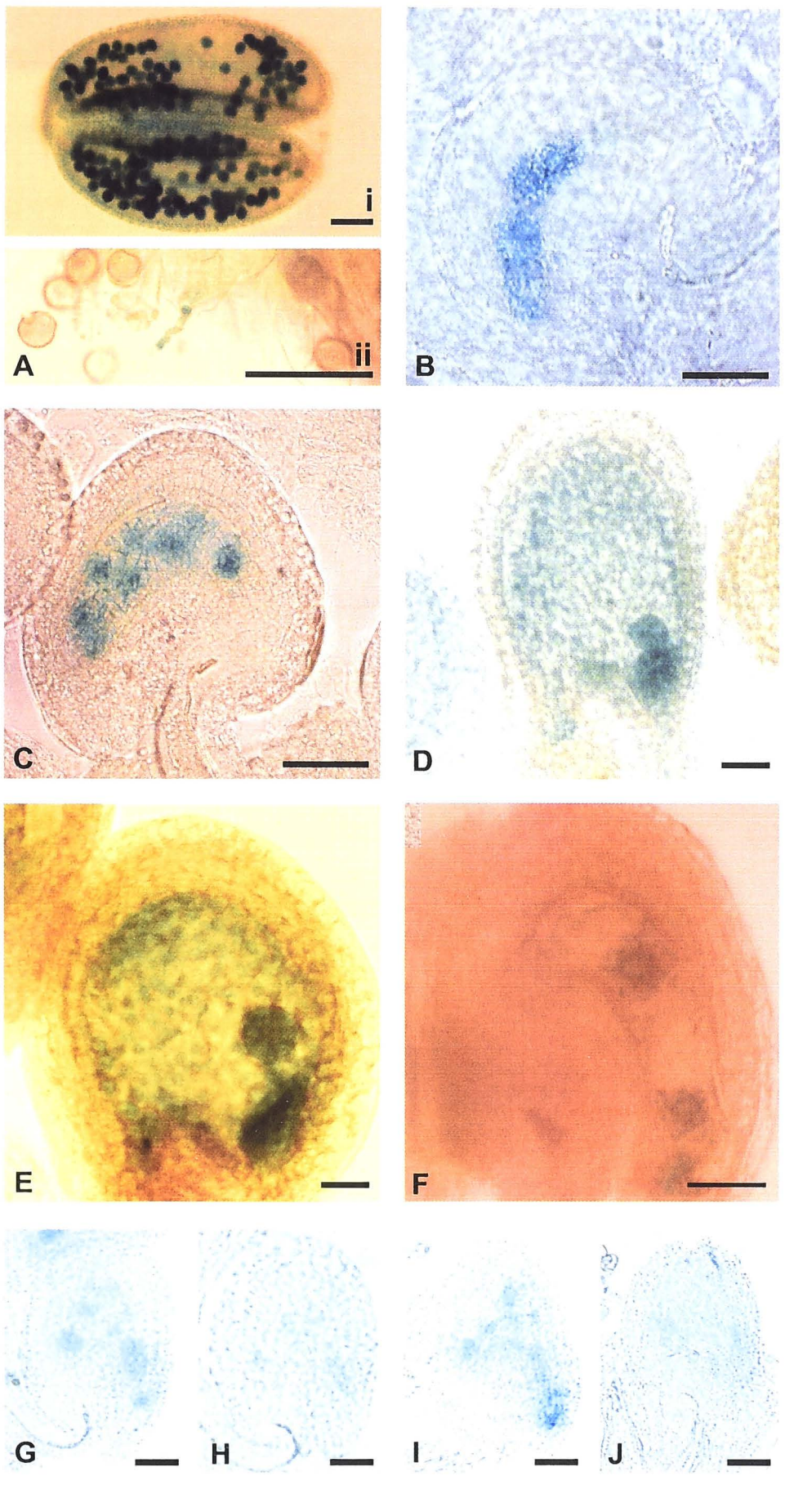


Fig. 4.5 *AtWRKY10::GUS* activity. (A i) Mature pollens showing GUS activity in a homozygous transgenic plant. (A ii) Pollen germination on L*er* stigma, showing pollen tube blue. (B) *AtWRKY10::GUS* ovule showing GUS activity in possible two endosperm nuclei 12 hours post fertilization. (C) *AtWRKY10::GUS* ovule showing GUS activity in 8 endosperm nuclei 24 hours post fertilization. (D) *AtWRKY10::GUS* ovule showing GUS activity in endosperm nuclei and globular embryo 72 hours post fertilization. (E) *AtWRKY10::GUS* ovule showing GUS activity in endosperm nuclei and globular embryo 96 hours post fertilization. (F) L*er* ovule showing GUS activity in 2 endosperm nuclei 24 hours post fertilization by *AtWRKY10::GUS* plants. (G & H) L*er* ovule showing higher GUS activity if pollinated with *min3* (G) than pollinated with *MIN3* (H). (I & J) *min3* ovule showing higher GUS activity if pollinated with *min3* (I) than pollinated with *MIN3* (J). (bar=0.05mm)

reciprocal experiment, when *AtWRKY10::GUS* was introduced from pollen into either L*er* or *min3/min3* mutant plants, ovules of L*er* pollinated with *AtWRKY10::GUS* pollen showed lower GUS activity than those of *min3/min3* pollinated with the same *AtWRKY10::GUS* pollen. When a *min3/min3* plant carrying *AtWRKY10::GUS* was pollinated with either *min3* or L*er* pollen the *min3/min3/min3* endosperm showed stronger GUS activity than seen in *min3/min3/MIN3* endosperm (Fig. 4.5 I & J).

These results indicate that the expression of *AtWRKY10:GUS* is inversely related to the dosage of the *MIN3* allele and is consistent with the model that *MIN3* represses its own expression. Several other *WRKY* genes are known to positively or negatively regulate their own expression (Robatzek and Somssich., 2002; Chen and Chen., 2002).

4.4 Discussion

4.4.1 *Arabidopsis* has a small number of genes controlling seed size.

Plant seed is generated via a complex developmental process. It contains maternal sporophytic tissue, the integuments harboring the female gametophyte. Following fertilization, the diploid embryo and triploid endosperm are produced. Seed development requires an interplay of zygotic, maternal and gametophytically operating genes. The genes described here, *MIN2* and *MIN3*, control seed size and have a sporophytic action in the developing seed.

Previously described mutants *iku1* and *iku2* (Garcia et al., 2003) have phenotypes similar to those of *min2* and *min3* mutants. *iku2* mapped to

the same chromosomal region as *min2*. Complementation analysis will determine if *iku2* and *min2* are, in fact, allelic. *MIN3* maps on chromosome 1 and is a different locus to either *iku1* or *iku2*.

Garcia et al (2003) reported that in a further mutational analysis they isolated two other mutant alleles of *iku1* and one allele of *iku2* implying that their mutational analysis was reaching saturation and that only a small number of genes might be concerned with the size of the mature seed.

The three seed size mutants have similar phenotypes and may operate in a single developmental pathway. Their products could interact in a protein complex, facilitating endosperm development with all three products required for normal seed development. The *MIN3* locus maps to a location similar to a major seed size QTL identified in crosses between L*er* and Cape Verde ecotypes (Alonso-Blanco et al., 1999). The two other loci on chromosomes 1 and 3 could correspond to other QTLs for seed size.

Garcia et al (2003) looked for interactions between the *iku* mutants with other loci which were known to have either sporophytic or gametophytic modes of action. The sporophytic recessive mutants, *knolle*, *spätzle*, and *hallimasch,* display defects in endosperm cellularisation (Lauber et al., 1997; Sørensen et al., 2002). Mutant *knolle* affects both cytokinesis in the embryo and cellularisation of the endosperm as a result of the loss of function of the syntaxin *KNOLLE*. Mutant *spz* is characterized by the absence of cellularisation only in the endosperm and viable embryo. Mutant *hallimasch* is characterized by the complete absence of microtubules in the embryo and in the endosperm, leading to the

generation of large nuclei in the endosperm and arrested embryos with a few multinucleate cells. In the double mutant genotypes with *knolle*, *spätzle* and *hallimash*, the *iku* mutants had additive phenotypes, without increasing seed size in comparison with *iku* seeds. They concluded that the small seed size phenotype could be produced by pathways other than by modification of endosperm cellularisation.

The *FIS2* gene has a gametophytic action in endosperm development and, in the pollinated *fis2* mutant, cellularisation of the endosperm does not occur. In the double mutants both the *min* and *fis2* phenotypes were retained. A similar result was obtained by Garcia et al (2003) in the double mutants of *iku1* and *iku2* with *fis1*. Both analyses show that the reduction in seed size of *min*2, *min*3, *iku*1 and *iku2* , which all have a sporophytic mode of action, was independent of the action of *FIS* genes with their gametophytic mode of action.

4.4.2 Molecular analyses of the *MIN3* gene

MIN3 is a transcription factor of the *WRKY* class and specifically corresponds to the *WRKY10* gene (Eulgem et al., 2000). There are about 80 *WRKY* genes in *Arabidopsis*. This class of transcription factor appears to be restricted to the plant kingdom. WRKY gene products form homo and hetero dimers and are known to regulate loci involved in pathogen response, in storage protein deposition and in the regulation of α-amylase in germination (Eulgem et al., 2000). One *WRKY* gene, *TTG2*, is involved in the development of the trichomes and seed coat (Johnston et al., 2002). Some *WRKY* genes have been shown to be auto regulatory (Robatzek and Somssich., 2002; Chen and Chen., 2002). The WRKY binding motif (W-box) with a consensus sequence of (T)TGAC(C/T) has

been identified in the promoters of both the auto regulatory *WRKY* genes and their target genes. We have shown that *MIN3* is likely to be an autoregulatory *WRKY* gene with W box motifs in the promoter. The genes defined by the other small seed mutations such as *min2* or *iku1* could be other WRKY class genes or target genes of *MIN3*.

4.4.3 Autorepression of *MIN3*

Expression of *MIN3::GUS* is higher when it is introduced into developing *min3/min3* endosperm than in *min3/MIN3* or *MIN3/MIN3* endosperm lending support to the idea that *MIN3* gene expression is repressed by *MIN3*. Consistent with this suggestion is the finding of three W boxes (WRKY binding sites) in the promoter.

Another WRKY class gene, *AtWRKY18,* contains a cluster of *WRKY* binding sites in its promoter which act as negative regulatory elements for the expression of the gene (Chen and Chen., 2002). *MIN3/AtWRKY10* belongs to this class of autorepressive *WRKY* gene which could regulate itself and other *WRKY* genes to control key aspects of endosperm development, such as onset of cellularisation.

4.4.4 Paternal and zygotic expression and lack of imprinting of the *MIN3* locus

A large number of genes expressed specifically in endosperm have been shown to be imprinted in the male genome and not expressed in early seed development (Vielle-Calzada et al., 2000). This is not the case for *MIN3*.

The *MIN3* gene is expressed in the male gamete (pollen) but not in the female gamete (embryo sac) before fertilization. After fertilization both paternally and maternally derived *MIN3* genes are expressed early in the endosperm from the two nucleate stage to cellularisation and at a lower level in the embryo from the globular to the early heart stage of development. Thus *MIN3* shows paternal gamete specific expression and no genomic imprinting.

4.4.5 *MIN3* gene and genomic conflict

MIN3-type genes might play a role in mediating genomic conflict during seed development. It has been suggested that *FIS* genes are maternal-specific genes involved in the genomic conflict that occurs during endosperm development (Chaudhury et al., 2001; Baroux et al., 2002). The female gametophytic specific expression of *FIS* genes, their paternal imprinting and the ability of wild type *FIS* to suppress the proliferation of endosperm have been suggested to indicate a role of *FIS* genes in reducing seed size, and thereby acting as a maternal conflict gene (Haig., 1999).

In contrast, the pollen specific expression of *MIN3*, its lack of paternal imprinting and seed size promoting role of *MIN3* suggests that this class of gene might be a mediator of paternal conflict. Thus *MIN* and *FIS* genes might repress each other, a possibility that has not yet been tested.

CHAPTER 5

General discussion

5.1 The role of *FIS* class genes in seed development

The *FIS* class genes, *MEA/FIS1*, *FIS2*, and *FIE/FIS3* have specific functions in *Arabidopsis* seed development. The *FIS* class genes prevent the central cell nucleus from progressing into endosperm prior to fertilisation and regulate endosperm proliferation and polarity after fertilisation (Chaudhury et al., 1997, Ohad et al., 1997). The loss of function of any one of them causes the failure of repression of the nucleus division of the central cell and leads to diploid autonomous endosperm growth up to the stage of cellularisation in unfertilised ovules of *mea/fis1* and *fis2*. All the *fis* mutant ovules, when fertilised with either wild type or *fis* mutant pollen, give aborted seeds with overproliferated, polarity-disturbed and uncellularised endosperm. Wild type ovules fertilised with *fis* pollen give wild type seeds. Thus the reciprocal crosses between *fis* mutants and wild type give different results, indicating that *FIS* genes exert a parent-of-origin effect on seed development. A parent-of-origin effect has also been observed in reciprocal crosses of plants with different levels of ploidy or with different levels of DNA-methylation.

The DNA sequences of *FIS* genes suggest their molecular functions in seed development (Luo et al., 1999; Ohad et al., 2000). MEA/FIS1 and FIE/FIS3 are related to two classes of polycomb group proteins, *Enhancer of zeste* and *Extra sex combs*, respectively, which initially were identified as partners of a complex that repressed homeotic gene

expression in *Drosophila* (Gutjahr et al., 1995; Jones et al., 1993). FIS2 is a C2H2 Zinc finger DNA binding protein and shows a distinctive repeat structure. A role for FIS2 in chromatin remodeling may be inferred from its homology to a *Drosophila* polycomb group protein, Suppressor of Zeste 12 (Su(z)12) (Birve et al., 2001). Thus *FIS* genes like their polycomb counterparts in different organisms act as repressive modifiers of chromatin by the formation of multiprotein complexes that may keep target genes inaccessible to the transcriptional machinery.

The maternal expression and paternal imprinting of all *FIS* class genes sheds light on the roles of *FIS* genes in seed development. *In situ* hybridization studies have shown that *MEA/FIS1* mRNA is present, before fertilization, in the eight-nuclei embryo sac, in the egg cell, and in the central cell (Vielle-Calzada et al., 1999). After fertilization, *MEA/FIS1* mRNA was detected in all cells of the suspensor and the embryo until the heart and torpedo stage of embryo development. *MEA/FIS1* mRNA was also located in free endosperm nuclei but was absent from cellularised endosperm. In this work, I analysed the *MEA/FIS1, FIS2* and *FIE/FIS3* expression by fusing the promoter of these three genes to *GUS*. I detected *GUS* expression in the unfertilised central cell nucleus and the dividing endosperm nuclei in non-cellularised endosperm in *MEA/FIS1::GUS*. *FIS2::GUS* showed a similar expression pattern to *FIS1/MEA::GUS*: *FIS2::GUS* was detected in the unfertilised central cell nucleus and in the dividing endosperm nuclei in non-cellularised endosperm. *FIE/FIS3*, is expressed in both sporophytic and gametophytic tissues: cauline leaf, stem, root, immature floral buds, open flower, and young siliques, as shown by reverse transcriptase-PCR of RNA pools derived from various tissues (Ohad et al., 1999). Using the *FIE/FIS3::GUS* reporter approach, I found that *FIE/FIS3* was expressed

in the unfertilised ovules with a dense GUS activity located in the central cell nucleus. After fertilisation, *FIE/FIS3::GUS* was detected in both non-cellularised and cellularised endosperm, and heart or later stage embryos. *FIS3/FIE::GUS* was also detected in various sporophytic tissue. Yadegari et al 2000 obtained similar results using *FIE/FIS3::GFP* (and *GUS*) transgenic lines. My finding of expression of all the *FIS* genes in the central cell and endosperm support the concept that all the FIS proteins work in the same complex.

The expression pattern of all the *FIS* genes in the unfertilised central cell nucleus is consistent with a role in repressing endosperm development. The expression of the *FIS* genes in dividing endosperm also suggests that they suppress endosperm development because the endosperm is over-proliferated in all three mutants. *mea/fis1* and *fis2* mutants can be made as homozygotes and no other morphological phenotypes are detected, indicating that *MEA/FIS1* and *FIS2* might function solely in the endosperm. The endosperm-specific expression of *MEA/FIS1::GUS* and *FIS2::GUS* supports this conclusion. *FIE/FIS3* activity can be detected in the endosperm tissue but is also present in some other sporophytic tissues, indicating that *FIE/FIS3* has a role in multiple tissues rather than being restricted to endosperm. I was unable to obtain *fie/fie* homozygous mutants, as were other groups working in this field. The lack of *fie/fie* homozygotes, and the paternal and maternal expression of *FIE/FIS3::GUS* in the embryo indicate that *FIE/FIS3* functions in the embryo as a sporophytic gene. Thus *FIE/FIS3* causes a parent-of-origin effect in endosperm but a sporophytic effect in the embryo. Further, Kinoshita et al. (2001) reported that FIE-mediated polycomb complexes are an essential component of a floral repression mechanism established early during plant development. The loss of *FIE* function can cause the

seedling shoot to produce flower-like structures and organs. Katz et al. showed that FIE/FIS3 interact with CURLY LEAF, another E(z) homolog in *Arabidopsis*, and the *FIE/FIS3* co-suppressed plants displayed dramatic morphological aberrations, similar to those exhibited by plants overexpressing *AGAMOUS* (*AG*) or *CURLY LEAF* mutants. FIE/FIS3 also interact with EZA1, one of three E(z) homolog in *Arabidopsis* (Luo et al, 2000), demonstrate that FIE/FIS3 is a versatile protein that interact in different SET domain E(Z) proteins, results in differential regulation of gene expression throughout the plant life cycle.

MEA/FIS1 and *FIE/FIS3* have been shown to be imprinted; during early seed development only the maternal allele is expressed. Vielle-Calzada et al. (1999) showed that in the triploid endosperm nuclei only the two maternal copies of *MEA/FIS1* locus were expressed, indicating that the paternal copy was silenced. They confirmed the silencing of the paternal *MEA/FIS1* copy in 54 hour old seeds. Kinoshita et al. (1999) showed similar results for the imprinting of *MEA/FIS1*. They found that only the maternal *MEA/FIS1* mRNA was detected in the endosperm from seeds at the torpedo stage and later. The expression of both maternal and paternal *MEA/FIS1* alleles was observed in the embryo of the same age and other sporophytic tissues such as leaf, stem, and root. In general, their expression data fit with earlier genetic observations in which reciprocal crosses involving wild-type and mutant alleles of the three *FIS* genes did not give identical results, indicating a parent-of-origin effect (Chaudhury et al., 1997; Ohad et al., 1997; Grossliknaus et al., 1998).

Yadegari et al. 2000 observed *FIE/FIS3* paternal imprinting by analysing *FIE/FIS3*::*GFP* (and *GUS*) transgenic lines. I investigated the paternal imprinting of the three *FIS* genes using *FIS* gene promoters::*GUS* lines

(Luo et al., 2000). *FIS2* is completely imprinted during endosperm development. The paternal copies of *MEA/FIS1* and *FIE/FIS3* are silenced in early endosperm but the silencing is de-repressed later. Both paternally and maternally derived copies of *FIE* were expressed in the embryo and did not show imprinting in this tissue. Moreover, the maternal expression pattern and paternal imprinting of all *FIS* genes were not altered in conditions of DNA hypomethylation, indicating that the imprinting of *FIS* class genes was not mediated by DNA methylation (Luo et al., 2000).

5.2 The role of hypomethylation in seed development

The reciprocal crosses between a *fis* mutant and a wild type, between a polyploid and a diploid, or between a hypomethylated plant and a wild type each produce different types of seeds depending on the direction of the crosses. *fis* ovules always give aborted seeds with over-proliferated endosperm when pollinated with wild type pollen and wild type ovules always give viable seeds when pollinated with *fis* pollen. Diploid *Arabidopsis* produced bigger seeds when pollinated with tetraploid pollen than when pollinated with diploid pollen, indicating that the increase of paternal gene dosage in tetraploid pollen resulted in bigger seeds (Scott et al., 1998). Hypomethylated pollen resulted in small seeds in wild type ovules, indicating a decrease of paternal gene dosage in hypomethylated pollen (Adams et al., 2000; Luo et al., 2000). When the diploid was pollinated with a hexaploid, the endosperm over proliferated and did not cellularise (Scott et al., 1998), similar to the endosperm in the *mea/fis1, fis2* and *fie/fis3* mutant. So in the *fis* mutants there is a similar parental gene-dosage-dependent effect causing endosperm over-proliferation as when polyploid pollen is used for fertilisation.

The over proliferation of the *mea/fis1*, *fis2* and *fie/fis3* endosperm can be reduced when the *fis* ovules are fertilised with hypomethylated pollen, while the normal proliferation of the wild type endosperm can also be reduced when the wild type ovules are fertilised with hypomethylated pollen. This occurs without activating the paternal *FIS* copies as shown in chapters 2. The *mea/fis1* and *fis2* maternal defects could be rescued with the hypomethylated *mea/fis1* and *fis2* pollen. Therefore, there are genes that have dosage effects in endosperm development and are regulated both by the FIS complex and by DNA methylation. In chapter 3, the genetic mapping showed that there were at least two *Paternal Modifiers of FIS* (*PMF*) loci for *fis1/mea* and three *PMF* loci for *fis2* that were modified by DNA hypomethylation and acted as epi-alleles that were able to rescue the *fis* defect. It is likely that the *PMF*s were epi-marked by the hypomethylation and resulted in the rescue of the *fis* maternal defect when introduced into the paternal hypomethylated genome. The nature of these genes is still to be identified. Kohler et al (2003) showed that the MADS-box gene *PHERES1* (*PHE1*) is deregulated in the *fis*-class mutants. *PHE1* expression is epigenetically regulated by PcG proteins and hypomethylation. Interestingly, one *PMF* locus is close to the location of *PHE1*, indicating *PHE1* is possibly one of the *PMFs*. *PHE1* and other *PMF*s might be regulated by both *FIS* genes and DNA methylation and involved in mediating parent of origin effect in seed development.

5.3 The role of *MIN3/AtWRKY10* in seed development

As pollination with a paternal hypomethylated genome results in small seed size, I assumed that some of the genes controlling seed size might be

regulated by DNA methylation. In the screening for mutants with small seed size, *min3* and *min2* were isolated. The previously described mutants *iku1* and *iku2* (Garcia et al., 2003) have phenotypes similar to those of *min2* and *min3* mutants; a reduced number of endosperm nuclei and early cellularisation of the endosperm in the developing seed. *iku2* mapped to the same chromosomal region as *min2*,and *iku2* and *min2* could be allelic. *MIN3* maps to chromosome 1 and is a different locus to either *iku1* or *iku2*. The three seed size mutants (*iku1*, *iku2/min2* and *min3*) with similar phenotypes may operate in a single developmental pathway. The *MIN3* locus maps to a location close to a major seed size QTL identified in crosses between L*er* and Cape Verde ecotypes (Alonso-Blanco et al., 1999).

MIN3 is a *WRKY* class transcription factor (Eulgem et al., 2000). The WRKY proteins belong to a superfamily of transcription factors with up to 100 members in *Arabidopsis*. Although the information on their precise regulatory functions are limited, they appear to be involved in the regulation of various physiological programs, including pathogen defence, senescence and trichome development (reviewed by Eulgem et al., 2000). The fact that these *WRKY* genes appear to be specific to plants suggests that they play an important role in plant development.

In this study, I demonstrated that *AtWRKY10* controls early endosperm development. The mutation in *AtWRKY10* causes precocious endosperm cellularisation and lower endosperm content. Thus, *AtWRKY10* and *FIS* class genes have opposite functions on endosperm proliferation. The *FIS* class genes are repressors of the central cell nucleus division and endosperm development. The loss of function of any of them leads to formation of aborted seeds with overproliferated and uncellularised

endosperm in fertilised ovules. In contrast to the *FIS* genes, the mutation in *AtWRKY10* causes a reduction of endosperm content and precocious cellularisation and leads to the formation of seed with small size and weight.

In the double mutants between *min* and *fis2* both the *min* and *fis2* phenotypes were retained. A similar result was obtained by Garcia et al (2003) in the double mutants of *iku1* and *iku2* with *fis1*. Both analyses show that the reduction in seed size of *min*2, *min*3, *iku*1 and *iku2* , which all have a sporophytic mode of action, was independent of the action of *FIS* genes with their gametophytic mode of action. This result implies that *MIN* and *IKU* are not the targets of DNA methylation.

5.4 A model for the functions of the FIS complex and DNA methylation in seed development

In this model, *FIS* class genes are expressed maternally to repress the downstream genes, *Paternal Modifiers of FIS* (*PMF*s) that mainly trigger and promote endosperm development. The maternal copies of *PMF*s are silenced due to the *MEA/FIS1*, *FIS2* and *FIE/FIS3* repression in the wild type plants, and the paternal copies are, therefore, active and responsible for triggering the endosperm development when brought in by pollination. As a result, the endosperm development is normal in wild type. In the *mea/fis1, fis2* and *fie/fis3* mutants, the maternal copies of *PMF*s become active because of the ~~failure~~ lack of repression and so the endosperm develops autonomously even without pollination. It should be noted that *mea/fis1* and *fis2* autonomous endosperm can become cellularised as in the wild type plant. However, when these mutants are pollinated with wild type pollen, both maternal and paternal copies of

*PMF*s are active and thus the increased dosage of *PMF*s may cause endosperm over-proliferation similar to ~~that observed in~~ the diploid pollinated with the hexaploid pollen ~~because there are too many~~ paternal copies of *PMF*s ~~from hexaploid pollen~~. These *PMF*s are also regulated by DNA methylation and confer a gene dosage effects on endosperm development. Under the paternal hypomethylation condition, I suggest that the paternal copies of *PMF*s are down-regulated by activating *FIS* like repressors due to demethylation, or ~~even~~ by direct hypermethylation of paternal copies of the *PMF*s ~~themselves~~. Thus the wild type plants give small seeds when pollinated with hypomethylated pollen because of the ~~insufficient~~ dosage of paternal copies of the *PMF*s. *mea/fis1, fis2* and *fie/fis3* mutants give normal seeds when pollinated with the hypomethylated pollen due to the restored balance between the active maternal copy of *PMF*s and the inactive paternal copy of *PMF*s.

This model also explains why seed developing from interploidy crosses often show different seed phenotypes depending on which parent was the female or male. Analysis of progeny from interploidy crosses in many plants species including *Arabidodpsis* (Scott et al., 1998), reveals that paternal genomic excess is associated with overproduction of endosperm, whereas maternal genomic excess is associated with endosperm reduction. The effects on seed development of changing the ratio of maternal to paternal genomes may be due to change in the ratio of maternal to paternal copies of imprinted genes, including *FIS* genes, *Paternal Modifiers of FIS*s (*PMF*s), and other unknown paternally or maternally imprinted genes. DNA methylation participates in the regulation of some maternally or paternally imprinted genes and thus mediates the parent-of-origin effect. *PMF*s are possibly regulated by DNA methylation.

This model supports the parental conflict theory that explains the existing genomic imprinting in flowering plants and mammals (Haig and Westoby., 1989; 1991). During fertilization, a mother may have offspring by more than one father. The embryos acquire a significant amount of resources from maternal tissues. As a result, the paternal genome strives to extract the maximal amount of resources for their own offspring, whereas the maternal genome endeavours to allocate an equal amount of resources among offspring. During evolution, the imprinting arose from the conflict between maternal and paternal genomes. Genes tending to increase nutrient flow to the embryo and promote endosperm growth would be preferentially expressed by the paternal alleles (potentially *PMF*s), and the maternal allele would be silenced. By contrast, genes tending to restrict nutrient flow to the embryo and suppress endosperm growth would be preferentially expressed by the maternal alleles (Such as *FIS* genes), and the paternal allele would be silenced. Results from both genetic and molecular analyses of *FIS* genes support the parental conflict theory.

It seems that *MIN3/AtWRKY10* might also mediate parental conflict, because *AtWRKY10* activity is paternally specific before fertilisation. During endosperm development, both maternal copy and paternal copy are active to promote endosperm development.

CHAPTER 6

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